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TABLE OF CONTENTS

NO. 1, JULY, 1914

An Attempt to Transmit Poliomyelitis by the Bite of <i>Lyperosia Irritans</i> <i>Edvard Francis</i> - - - - -	1
Complement-Fixation in <i>Gonococcus</i> Infections <i>John A. Kolmer and Claude P. Brown</i> - - - - -	6
A Simple Method of Cultivating Bacilli, Preferring Conditions of Partial Anaerobiosis (<i>B. Abortus</i> , Bang; <i>B. Bifidus</i> , Tissier) <i>George D. Horton</i> - - - - -	22
An Attenuated Culture of <i>Trypanosoma Brucei</i> <i>Charles August Behrens</i> - - - - -	24
Classification of Pathogenic Streptococci by Fermentation Reactions <i>J. G. Hopkins and Arvilla Lang</i> - - - - -	63
Anaerobic Cultures in Scarlet Fever (<i>with Plate 1</i>) <i>George F. Dick and Gladys R. Henry</i> - - - - -	85
The Characteristics of Bacteria of the Colon Type Found in Bovine Feces <i>L. A. Rogers, William Mansfield Clark, and Alice C. Evans</i> - - -	99
The Toxins of Intestinal Obstruction <i>Fraser B. Gurd</i> - - - - -	124
The Relationship of Septic Sore Throat to Infected Milk <i>Joseph A. Capps and David J. Davis</i> - - - - -	130
Experimental Bovine Mastitis Produced with Hemolytic Streptococci of Human Origin <i>David J. Davis and Joseph A. Capps</i> - - - - -	135
An Investigation into the Fermentative Activities of the Aciduric Bacteria <i>Alfred H. Rahe</i> - - - - -	141
Purpura Associated with <i>Bacillus Mucosus</i> in the Blood <i>G. C. Weil and J. W. McMeans</i> - - - - -	151
Studies on the Sanitation of Swimming Pools <i>Wallace A. Manheimer</i> - - - - -	159
Studies on the Classification of the Colon Group <i>I. J. Kligler</i> - - - - -	187
The Quinin Treatment of Rabies <i>James Gordon Cumming</i> - - - - -	205
A Method for Making Carbohydrate Serum Broth of Constant Composition for Use in the Study of Streptococci <i>W. L. Holman</i> - - - - -	209
The Action of Vaccines and of Concentrated Antistreptococcus Serum in Experimental Streptococcal Arthritis <i>Josiah J. Moore</i> - - - - -	215
The Use of Decolorized Acid Fuchsin as an Acid Indicator in Carbohydrate Fermentation Tests with Some Remarks on Acid Production by Bacteria <i>W. L. Holman</i> - - - - -	227
Experiments on the Variability of the Fermentative Reaction of Bacteria, Especially the Streptococci <i>William C. Thro</i> - - - - -	234

No. 2, SEPTEMBER, 1914

Tuberculocidal Action of Certain Chemical Disinfectants. Studies on the Biochemistry and Chemotherapy of Tuberculosis. IX	
<i>Lydia M. DeWitt and Hope Sherman</i> - - - - -	245
The Standardization of Antihog-Cholera Serum	
<i>Thomas P. Haslam and O. M. Franklin</i> - - - - -	257
A Biometrical Study of the Mucosus Capsulatus Group	
<i>J. G. Fitzgerald</i> - - - - -	268
The Formation of Antibodies in Rats Fed on Pure Vegetable Proteins (Osborne-Mendel Stunting Food)	
<i>Ludvig Hektoen</i> - - - - -	279
The Bacteriology of Vaginitis	
<i>W. B. Sharp</i> - - - - -	283
The Relative Longevity of Different Streptococci and Possible Errors in the Isolation and Differentiation of Streptococci	
<i>W. L. Holman</i> - - - - -	293
Observations on the Growth of the Gonococcus and the Staphylococcus Albus from the Urethra in Plate Culture (A Criticism of Warden's Work)	
<i>E. G. Crabtree</i> - - - - -	309
Some Structural Transformations of the Blood-Cells of Vertebrates (<i>with Plate 2</i>)	
<i>G. L. Kite</i> - - - - -	319
Infection of Man with Bacterium Tularensis (<i>with Plate 3</i>)	
<i>William B. Wherry and B. H. Lamb</i> - - - - -	331
The Etiology of Dengue. An Attempt to Produce the Disease in the Rhesus Monkey by the Inoculation of Defibrinated Blood	
<i>C. H. Lavinder and Edward Francis</i> - - - - -	341
The Bacillus Abortivus Equinus as an Etiological Factor in Infectious Arthritis of Colts	
<i>Edwin S. Good and Wallace V. Smith</i> - - - - -	347
An Anaerobic Vibrio Isolated from a Case of Acute Bronchitis	
<i>Ruth Tunnicliff</i> - - - - -	350
A Pleomorphic Branching Organism Isolated from a Case of Chronic Rhinitis	
<i>Ruth Tunnicliff</i> - - - - -	352
The Esterase Activity of Plain and Dextrose Broth Cultures of the Typhoid Bacillus. Studies in Bacterial Metabolism. XXXIX	
<i>A. I. Kendall and J. P. Simonds</i> - - - - -	354
The Bacteriological and Chemical Evidence of the Occurrence of a Hexose Sugar in Normal Milk	
<i>Harry M. Jones</i> - - - - -	357
The Treatment of Tetanus by Antitetanic Serum	
<i>Ernest E. Irons</i> - - - - -	367
The Growth and Viability of Streptococci of Bovine and Human Origin in Milk and Milk Products	
<i>David John Davis</i> - - - - -	378
The Renal Changes in Rabbits Inoculated with Streptococci	
<i>E. R. LeCount and Leila Jackson</i> - - - - -	389
The Etiology of Pyemic Arthritis in Foals	
<i>Frank W. Schofield</i> - - - - -	409

TABLE OF CONTENTS

vii

No. 3, NOVEMBER, 1914

The Metabolism of Saprophytic Human Tubercle Bacilli in Plain, Dextrose, Mannite, and Glycerin Broths. Studies in Acid-Fast Bacteria. I	
<i>A. I. Kendall, A. A. Day, and A. W. Walker</i>	417
The Metabolism of Certain Rapidly Growing Human Tubercle Bacilli in Broth Free from Lipoids and Fatty Substances. Studies in Acid-Fast Bacteria. II	
<i>A. I. Kendall, A. A. Day, and A. W. Walker</i>	423
The Metabolism of Certain Rapidly Growing Human Tubercle Bacilli in a Modified Uschinsky Medium. Studies in Acid-Fast Bacteria. III	
<i>A. I. Kendall, A. A. Day, and A. W. Walker</i>	428
The Metabolism of Certain Rapidly Growing Tubercle Bacilli in Media with Inorganic Salts as Sources of Nitrogen. Studies in Acid-Fast Bacteria. IV.	
<i>A. I. Kendall, A. A. Day, and A. W. Walker</i>	433
The Metabolism of "Lepra Bacillus," Grass Bacillus, and Smegma Bacillus in Plain, Dextrose, Mannite, and Glycerin Broths. Studies in Acid-Fast Bacteria. V	
<i>A. I. Kendall, A. A. Day, and A. W. Walker</i>	439
The Occurrence of a Soluble Lipase in Broth Cultures of Tubercle Bacilli and Other Acid-Fast Bacteria. Studies in Acid-Fast Bacteria. VI	
<i>A. I. Kendall, A. W. Walker, and A. A. Day</i>	443
The Relative Activity of the Soluble Lipase and Lipase Liberated during Autolysis of Certain Rapidly Growing Tubercle Bacilli. Studies in Acid-Fast Bacteria. VII	
<i>A. I. Kendall, A. W. Walker, and A. A. Day</i>	451
Observations on the Specificity and Thermostability of the Lipase Developed during the Growth of a Rapidly Growing Tubercle Bacillus in Media of Varied Composition. Studies in Acid-Fast Bacteria. VIII	
<i>A. I. Kendall, A. W. Walker, and A. A. Day</i>	455
A Comparison of the Curves of Lipolytic Activity and Proteolysis of Certain Rapidly Growing Human Tubercle Bacilli in Media of Varied Composition. Studies in Acid-Fast Bacteria. IX	
<i>A. I. Kendall, A. W. Walker, and A. A. Day</i>	460
A Comparison of the Curves of Lipolytic Activity and Proteolysis of Certain Acid-Fast Bacilli in Nutrient Broths. Studies in Acid-Fast Bacteria. X	
<i>A. I. Kendall, A. W. Walker, and A. A. Day</i>	467
The Effect of Cholesterol on Phagocytosis	
<i>Kaethe Dewey and Frank Nuzum</i>	472
The Formation of Chlamydospores in <i>Sporothrix Schenckii</i>	
<i>David John Davis</i>	483
An Experimental Study of the Influence of Iodin and Iodids on the Absorption of Granulation Tissue and Fat-Free Tubercle Bacilli. Studies on the Biochemistry and Chemotherapy of Tuberculosis. X	
<i>Edwin Frederick Hirsch</i>	487
The Antigenic Properties of Glycoproteins	
<i>Chester H. Elliott</i>	501
The Therapeutic Value of Copper and Its Distribution in the Tuberculous Organism. Studies on the Biochemistry and Chemotherapy of Tuberculosis. XI	
<i>Harry J. Corper</i>	518

The Relation between the Allergic Intracutaneous Reaction and the Symptoms of Anaphylaxis	
<i>Grace L. Meigs</i> - - - - -	541
The Protein Poison of the Tonsil	
<i>W. H. Burmeister</i> - - - - -	549
Histologic Differentiation by Means of Anilin Stains in Association with "Regressive Mordanting," with Special Reference to Elastic Tissue	
<i>H. F. Harris</i> - - - - -	561
The Effect of Gentian Violet on the Bacillus Tetani, Tetanus Toxin, and Certain Laboratory Animals	
<i>Ivan C. Hall and Loren B. Taber</i> - - - - -	566
The Production of Acid by the Bacillus Coli Group	
<i>William W. Browne</i> - - - - -	580

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No. 1

AN ATTEMPT TO TRANSMIT POLIOMYELITIS BY THE BITE OF *LYPEROSIA IRRITANS* *

EDWARD FRANCIS

(From the Laboratory of the Public Health Service, U. S. Marine Hospital, Savannah, Ga.)

Owing to the successful transmission of poliomyelitis from monkey to monkey through the bite of *Stomoxys calcitrans* reported by Rosenau and Brues and later by Anderson and Frost, it occurred to the writer, while investigating an epidemic of poliomyelitis in Texarkana and the vicinity in June and July, 1913, that possibly *Lyperosia irritans* might be similarly concerned in the transmission of that disease, because this blood-sucking fly prevailed in great numbers on the farms about Texarkana.

The idea was tested in experiments made at the United States Marine Hospital, Savannah, Ga., in August and September, 1913, and the results are here reported.

The poliomyelitis virus employed was obtained from the Hygienic Laboratory in Washington, and consisted of portions of the cervical and lumbar cord of a macacus rhesus monkey which had succumbed with typical symptoms of the disease July 26, 1913. Macacus rhesus monkeys were used in these experiments.

* Received for publication November 4, 1913.

Lyperosia irritans is a blood-sucking fly found in great numbers on cattle. Its most striking gross characteristic is the head-downward position which it assumes on a cow. The position is well illustrated in



FIG. 1.—*Lyperosia irritans* in a cage, showing head-downward position.

the accompanying photograph, which, while it was taken of the flies in a cage, is equally illustrative of the position which they take on the neck, shoulders, and sides of a cow. Another striking characteristic of these flies is their predilection for black cattle. Almost none are found on the white and yellow animals. The flies used in these experi-



FIG. 2.—Fly cage, over which monkeys were placed

ments were collected in a net from the cows which had just been admitted to the barn for milking in a dairy at Savannah, Ga. Before starting the experiment it was thought that *Stomoxys* would be found mixed in with *Lyperosia*, but fortunately only four *Stomoxys* were found among almost 6,000 *Lyperosia* collected. Great difficulty was experienced in keeping the flies alive in captivity. If left unfed for

twenty-four hours practically all of the flies die. It was found that frequent feeding on a live animal was necessary.

The plan finally adopted was to allow them to bite a monkey every six hours. The type of cage in which the flies were kept is shown in the illustration. After being shaven on the belly and insides of the thighs, the monkey was stretched over the wire gauze through which the flies fed.

EXPERIMENT 1.

SHOWING DATES AND HOUR OF EXPOSURE OF MONKEYS 1, 2, 3 AND 4, TO THE BITES OF THE FLIES

		August											Sept.
		21	22	23	24	25	26	27	28	29	30	31	1
Fly Cage A.	6 A. M.	..	1	1	1	1	1	4	4	4	4	2	2
	12 M.	..	2	2	2	2	2	2	2	2	2	2	..
	6 P. M.	..	1	1	1	1	1	1	4	4	2	2	..
	12 P. M.	..	1	2	2	2	2	2	2	2
Number of Flies		1,050	300	300	250	250	150	150	150
Fly Cage B.	6 A. M.	2	2	2	2	2	2	2	2	3	3
	12 M.	3	3	3	3	3	3	3	3	3	..
	6 P. M.	2	2	2	2	2	2	2	3	3	..
	12 P. M.	3	3	3	3	3	3	3
Number of Flies		..	750	200	200	200	180	120	120	75

Cage A was stocked with flies August 21 at 4 p. m. The flies fed on a rabbit at 6 p. m.

Cage B was stocked with flies August 22 at 4 p. m. The flies fed on a rabbit at 6 p. m.

Monkeys were applied to the cages for an hour and a half every six hours each day.

Monkey 1 was inoculated intracerebrally with poliomyelitis virus August 21 at 1 p. m., and fed the flies in Cage A. He developed poliomyelitis August 25 followed by complete paralysis, and was chloroformed August 28.

Monkey 2 fed the flies in Cages A and B, thus affording the flies in Cage B an opportunity to become infected through Monkey 2.

Monkey 3 fed only the flies in Cage B; it was hoped thus to demonstrate that the flies in Cage B had become infected from Monkey 2.

Monkey 4 helped to feed the flies in Cage A after the death of Monkey 1, but developed a bloody diarrhea August 29 which proved fatal September 5, and at autopsy was shown to be due to a severe colitis.

EXPERIMENT 2.

SHOWING DATES AND HOUR OF EXPOSURE OF MONKEYS 5, 6, 7, 8, TO THE BITES OF THE FLIES

		September																						
		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24		
Fly Cage C	6 A. M.	..	6	5	5	5	6	7	6	7	6	7	6	6	6	8	8	6	8	8	8	8	..	
	12 M.	..	5	7	6	7	..	6	7	6	7	6	6	6	6	8	8	8	8	8	8	8	..	
	6 P. M.	..	6	5	5	5	7	7	6	7	6	6	8	8	6	8	8	8	8	8	..	
	12 P. M.	..	5	7	6	7	6	6	7	6	7	7	6	7	8	8	
Number of Flies		900	250	250	240	150	150	150	200	150	120	75	30	25	20	15	5		
Fly Cage D	6 A. M.	6	7	6	7	6	7	6	7	6	7	6	7	6	
	12 M.	5	5	5	..	7	6	7	6	7	6	7	
	6 P. M.	7	6	7	6	6	7	6	7	6	7	6	
	12 P. M.	5	5	5	7	7	6	7	6	7	6	
Number of Flies		..	760	200	200	150	150	100	100											

(Flies from both cages were put into Cage C September 15.)

Cage C was stocked with flies September 4 at 4 p. m. The flies fed on a rabbit at 6 p. m.

Cage D was stocked with flies September 5 at 4 p. m. The flies fed on a rabbit at 6 p. m.

Monkeys were applied to the cages for an hour and a half every six hours each day.

Monkey 5 was inoculated intracerebrally with poliomyelitis virus September 4 at 11 a. m. and fed the flies in both cages. He showed definite symptoms of poliomyelitis on September 8, and died September 9 at 11 a. m.

Monkey 6 fed the flies in both cages until September 16 when he was replaced by Monkey 8.

Monkey 7 fed the flies in both cages until September 15 when he was killed accidentally. Altho he had remained healthy, a piece of his cervical cord was inoculated intracerebrally into a fresh monkey which remained healthy.

Monkey 8 on September 16 began feeding the flies which had been condensed from both cages into Cage C.

The number and kinds of flies with which the cages were stocked in these experiments is shown below:

	Aug. 21	Aug. 22	Sept. 4	Sept. 5
<i>Lyperosia irritans</i>	1,050	750	900	760
<i>Musca domestica</i>	56	57	10	31
<i>Stomoxys calcitrans</i>	1	0	1	1

SUMMARY

Lyperosia irritans in Experiment 1 were allowed to bite a rhesus monkey twice daily for a period of six days from the time of inoculation with poliomyelitis virus until its death, and were allowed to bite a fresh rhesus monkey twice daily during the same period, and twice daily during the succeeding six days. This monkey remained well.

Lyperosia irritans in Experiment 2 were allowed to bite a rhesus monkey twice daily for a period of four days from the time of inoculation with poliomyelitis virus until its death, and were allowed to bite two fresh rhesus monkeys (6 and 7) twice daily during the above period, and during the succeeding eight and seven days, respectively. Monkey 6 remained healthy. Monkey 7 was accidentally killed at the end of the seven-day period. A portion of its cervical cord was inoculated into a fresh monkey which remained healthy.

The flies employed in Experiment 2 were allowed to bite a fresh rhesus monkey (8) three times daily for eight days beginning on the twelfth day from the first exposure of the flies to an infected monkey. Monkey 8 remained healthy.

Monkeys 2, 3, 6 and 8 were still healthy October 27, 1913.

COMPLEMENT-FIXATION IN GONOCOCCUS INFECTIONS *

JOHN A. KOLMER AND CLAUDE P. BROWN

(From the Laboratory of Experimental Pathology University of Pennsylvania, Philadelphia.)

Following the application of the principles of complement-fixation in the serum diagnosis of syphilis, the possibilities of this method as a means of diagnosis were soon realized and in a short space of time many infections were studied. In no instance, however, have results been secured comparable to the diagnostic value of the syphilis reaction. This is probably due to two main factors: (1) syphilis quickly becomes a general infection with a resulting extensive antibody formation, and (2) the antibody of the *treponema pallidum* or at least the more prominent of two possible antibodies, is characterized by its great affinity for lipoids in tissue extracts (antigens). This peculiar lipodophilic antibody or reagin is found with any degree of constancy in only two other infections, frambesia and leprosy. Pure culture antigens of the *treponema pallidum* have not the same value in the syphilis reaction as the ordinary lipoidal extracts, and results in the serum diagnosis of syphilis with these culture antigens are comparable in their inconstancy and weakness to reactions in other infectious diseases with their specific culture antigens, including the gonococcus complement-fixation test. If Wassermann and his co-workers had had a pure culture antigen of the *treponema pallidum* instead of fortunately but unwittingly selecting a tissue the great value of the syphilis reaction would not have been so quickly realized.

In 1906, Muller and Oppenheim¹ applied the complement-fixation test in the diagnosis of gonorrheal arthritis, using a culture of the gonococcus as antigen. To them, therefore, belongs the credit of having recorded the first complement-fixation test in gonococcus infection. A little later in the same year, Carl Bruch,² who applied the reaction in 3 cases of gonorrhea and with the serum of immunized rabbits, reported favorable results. In 1907 Meakins³ reported positive reactions in 3 cases of gonorrheal arthritis and was the first in America to make a report on the subject. Vannod,⁴ who studied the specificity of the reac-

* Received for publication January 4, 1914.

1. *Wien. klin. Wchnschr.*, 1906, 19, p. 894.

2. *Deutsch. Med. Wchnschr.*, 1906, 32, p. 1368.

3. *Johns Hopkins Hosp. Bull.*, 1907, 18, p. 255.

4. *Deutsch. Med. Wchnschr.*, 1906, 32, p. 1984.

tion with sera of rabbits immunized with gonococcus proteid and one of a meningococcus, reported that the meningococcus immune serum did not show complement fixation with gonococcus antigen, and vice versa, that gonococcus amboceptor was not found by meningococcus antigen. Wollstein,⁵ in a study of the biologic relationship of the gonococcus and meningococcus, reported results differing from those of Vannod. She found the bacteriolytic amboceptors in the sera of rabbits, immunized with these cultures, to be closely related and yielding fixation of complement with either antigen. Teague and Torrey⁶ followed with a very important communication showing that the differences in results of previous investigators were probably due in part to the use of single strains of the organisms in the preparation of antigens and immune sera. They emphasized the fact that the gonococcus belongs to a heterogeneous family and in attempting the diagnosis of gonorrheal infections by the complement-fixation method extracts of several different strains should be used. Naz Vannod,⁷ and later Watabiki,⁸ found that the gonococcus and meningococcus antibodies were quite specific for the homologous antigens in complement-fixation reactions.

Particular attention was drawn to the gonococcus complement-fixation test by the work of Schwartz and McNeal.⁹ These investigators emphasized the necessity of using polyvalent antigens, and their encouraging reports have stimulated renewed interest in this subject. They found that a positive reaction was not obtained if the infection was confined to the anterior urethra, and that a strong reaction was not to be expected before the fourth week of the infection and then only in acute cases with complications. They regard a positive reaction as indicating the presence, or rather activity in the body, of a focus of living gonococci, although a negative reaction does not exclude gonococcus infection. A positive reaction was secured in 31.4 per cent. of cases regarded clinically as gonorrhea; in 54.8 per cent. of cases of chronic prostatitis giving a positive history of infection within three years; in 13.2 per cent. of cases regarded as clinically cured for at least three months. They secured positive reactions in a certain number of cases, especially in women, when the bacteriological examination failed to show gonococci. The test therefore has a more positive than negative value. With Flexner's antimeningococcus serum positive reactions resulted with their gonococcus antigen—with sera from cases of cerebrospinal meningitis (meningococcus) the results were negative.

In the following years numerous reports, including those of Swinburne,¹⁰ Gradwohl,¹¹ O'Neil,¹² Gardner and Clowes,¹³ have emphasized the practical value of the gonococcus complement-fixation test, particularly as an aid in determining whether or not a patient is cured.

Since the quantity of antibody formed in a strictly local gonococcus infection is probably small because of the comparatively slight cellular involvement, the complement-fixation reactions are generally weak and consequently require the closest technical attention especially in the preparation of antigen and in the accurate adjustment

5. *Jour. Exper. Med.*, 1907, 9, p. 588.

6. *Jour. Med. Research*, 1907, 17, p. 223.

7. *Centralbl. f. Bakteriöl.*, 1907, 44, p. 10.

8. *Jour. Infect. Dis.*, 1910, 7, p. 159.

9. *Am. Jour. Med. Sc.*, 1911, 143, p. 693; *ibid.*, 1912, pp. 144, 369 and 815.

10. *Arch. Diagnosis*, 1911, 4, 3, p. 227.

11. *Am. Jour. Dermat. and Syph.*, 1912, 16, p. 294.

12. *Boston Med. and Surg. Jour.*, 1912, 167, p. 464.

13. *New York Med. Jour.*, 1912, 96, p. 734.

of the hemolytic system. There can be no doubt of the truth of the contentions of Teague and Torrey, Schwartz and McNeil, that the more polyvalent the antigen the more satisfactory the complement-fixation tests. One of our interests in this subject was centered on methods of preparing antigens, and whether best results are secured by using an antigen composed essentially of the endotoxins of gonococci, or of the bacterial protein, or both.

The question of the rôle of secondary infection in chronic gonococcus infections of the genito-urinary tract is one of much interest. One of us having isolated a large number of cultures of staphylococci, streptococci, diphtheroid bacilli, etc., from cases of chronic urethritis and prostatitis, prepared antigens of these organisms and used them with all sera in an endeavor to determine their etiological relationship to these chronic processes, in so far as this could be done by complement-fixation reactions with homologous and mixed antigens.

Since the gonococcus, meningococcus, and the micrococcus catarrhalis possess morphological and biological characters in common, a study of their biological relationship by complement-fixation reactions with their various immune sera and antigens is of distinct importance as it has a direct bearing on the specificity of the gonococcus fixation test.

The objects of our study were mainly four-fold: (1) To ascertain the practical value of gonococcus antigen prepared after various methods; (2) to study the relation of mixed infection to chronic gonococcus infections of the genito-urinary tract by complement-fixation experiments with antigens of the organisms commonly found in mixed infections; (3) to study the practical value of the gonococcus fixation test in so far as it could be done with the material used in our investigations; (4) to study the biological relationship of the gonococcus to the meningococcus and the micrococcus catarrhalis by means of complement-fixation tests.

MATERIALS

Sera.—Of the 92 sera used, 73 were of persons giving positive bacteriological evidence of a clear history and definite clinical evidence of gonococcus infection, and nineteen were of persons giving a negative history and presenting no clinical evidences of infection. As gonorrhea is so widespread it was impossible to determine how many of these had had an infection at a remote and forgotten date. Five of these sera, however, were obtained from young boys in whom infection could be excluded with certainty.

Cultures.—The following cultures were used in preparing antigens: Eight strains of gonococci, all of which were originally secured from the Research Laboratory of the New York Board of Health; six strains of meningococci, five of which were secured from the Research Laboratory of the New York Board of Health, while the sixth was isolated from a case of meningitis by one of us; six strains of micrococcus catarrhalis, which were isolated from sputum and excised tonsils; three strains of staphylococcus albus and three of staphylococcus aureus, isolated from cases of chronic prostatitis; six strains of streptococci, isolated from cases of chronic prostatitis, and six strains of diphtheroid bacilli, isolated from cases of chronic prostatitis.

Antigens.—These were prepared as follows:

1. The washed gonococci were suspended in normal saline solution (unheated). Eight strains of gonococci were grown on serum agar for 4 days at 38 C. and growths washed off with sterile saline solution and centrifuged. The supernatant fluid was discarded and the sediment mixed with an excess of saline and centrifuged. The supernatant fluid was discarded and the sediment suspended in fresh sterile saline solution. To this suspension was added one-half of 1 per cent. phenol.

2. The washed gonococci were suspended in normal saline solution (heated). Eight strains were grown on blood agar, washed and suspended in Antigen 1. The emulsion was then heated at 60 C. for one hour and a preservative added.

3. Gonococcus antigen, purchased in the open market, was prepared after the following method:

Twenty-hour cultures on ascites agar in large flat quart flasks were washed off with physiological salt solution, shaken for eighteen hours, 0.2 per cent. trikresol added, and then passed through a Berkefeld filter. It was then diluted so that each 28 square inches of surface growth gave 16 c.c. of the antigen. This antigen was made of fourteen strains of the micrococcus gonorrhea.

4. Gonococcus antigen was prepared after the method of Besredka as modified by Gay: The cultures were grown on blood agar for 3 days, washed off with small amounts of saline solution, emulsion centrifuged, and sediment washed 3 times. Sediment was then suspended in sterile saline solution and treated with an equal part of absolute alcohol. After the precipitate had settled the supernatant fluid was drawn off and the sediment dried over sulphuric acid, accurately weighed, ground with crystals of sodium chlorid and made into a 2 per cent. suspension in normal saline solution. This stock dilution was further diluted as needed so that the actual amounts of dry antigenic substance contained in 1 c.c. were as follows:

1/40 dilution	=	0.5	mg.
1/80	"	0.25	"
1/160	"	0.125	"
1/320	"	0.062	"
1/640	"	0.031	"
1/1280	"	0.0155	" etc.

5. Alcoholic extract of washed gonococci: Seventy-two hour cultures were suspended in normal saline solution, centrifuged, and washed 3 times. The sediment was then treated with 95 per cent. alcohol, incubated at 38 C. for 3 days, shaken mechanically for 3 days more, and then passed through a Berkefeld filter.

The following antigens were prepared by growing cultures of the respective organisms on a serum media for 3 to 5 days, washing off the growths with sterile normal saline solution, centrifuging and washing the sediment 3 times,

suspending the washed sediment in normal saline solution, heating the emulsion to 60 C. for an hour, and adding trikresol as a preservative.

6. *Streptococcus* (6 strains).

7. *Staph. albus* (3 strains).

8. *Staph. aureus* (3 strains).

9. *Pseudo-diphtheria bacilli* (6 strains).

10. *M. catarrhalis* (6 strains).

11. *Meningococcus* (6 strains).

12. Mixed antigen composed of equal parts of Antigens 1-2-4-6-7-8-9-10 and 11.

Filtrates of Emulsions 2-6-7-8-9-10-11 and 12 were prepared as follows, and used as antigens: 5 to 25 c.c. of the antigens, depending on their density, were diluted with sufficient sterile saline solution to bring the total volume to 200 c.c. The diluted emulsions were then shaken mechanically for 72 hours and filtered through sterile Berkefeld filters.

No.	2 a.	Filtrate of	gonococcus	emulsion.
"	4 "	"	"	"
"	6 "	"	"	streptococcus "
"	7 "	"	"	<i>Staph. albus</i> "
"	8 "	"	"	<i>Staph. aureus</i> "
"	9 "	"	"	<i>B. pseudo-</i> <i>diphtheria</i> "
"	10 "	"	"	<i>M. catarrhalis</i> "
"	11 "	"	"	<i>meningococcus</i> "
"	12 "	"	"	mixed emulsions.

TECHNIC

(A) *Hemolytic System*: Both antisheep and antihuman hemolytic systems were employed, the latter as a control over the former to determine the influence of natural antisheep hemolysis in the human sera on the delicacy of the reactions.

With the antisheep system we used the same amounts as in our Wassermann technic, namely, one-half the amounts used in the original Wassermann method. Washed sheep's corpuscles were made up in a 2.5 per cent. suspension and used in doses of 1 c.c.; the fresh sera of guinea-pigs were diluted 1:20 and used as complement in dose of 1 c.c.; the hemolysin was titrated each day, and one hemolytic dose used.

All sera were heated to 55 C. for one-half hour and used in amounts of 0.5 to 0.2 c.c. Serum antigen and complement were incubated for one hour, amboceptor and corpuscles added, incubated for an hour or longer, depending on the hemolysis of the controls, and placed in the refrigerator over night. Readings were made next morning.

By titrating the hemolysin with each complement and corpuscle suspension the hemolytic system was accurately adjusted. The use of a larger amount than is usual in the technic of others is a matter of personal preference, sufficient amount of serum being easily obtained and requiring practically no more antigen. The controls were the same as we use in the routine Wassermann reaction: a serum control with each serum in maximum dosage; antigen, complement, hemolytic and corpuscle controls.

(B) *Antigen Titrations*: Two methods were used, (1) determining the anti-complementary dose of each antigen and using an arbitrary portion of this amount for the antigenic dose, and (2) titration with an antigenococcus immune serum. Of the two methods the former was found more satisfactory because the quan-

tity of gonococcus amboceptor in different immune sera is variable and of no great value in determining the proper dose for human sera, which may contain much less amboceptor.

The anticomplementary dose is easily determined at frequent intervals and if one-quarter or one-half of this amount is used in conducting tests with proper controls there is no danger of false reactions. Furthermore, this was the most feasible method in testing for an unknown amboceptor, as we were testing for staphylococcus, streptococcus, etc., amboceptor in the human sera.

The anticomplementary titrations of our antigens were conducted many times, the doses remaining constant throughout.

Antigen 4 became slightly anticomplementary in dose of 1 c.c. of dilution 1:320 (0.062 mg.). In many of the tests higher dilutions were used with 0.1 to 0.2 c.c. of serum, but with most sera we used but one dose of this antigen, 1 c.c. of dilution 1:1280 (.0155 mg.).

We adopted as our antigenic dose, one-quarter the amount shown by titrations to be anticomplementary. With this amount false reactions were avoided.

RESULTS WITH THE VARIOUS GONOCOCCUS AND OTHER ANTIGENS

The cases have been divided into four main groups: (1) gonorrheal urethritis of males; (2) probable gonococcus infection of women, many of whom came to operation; (3) acute vaginitis in young children; (4) control cases. All of these were submitted from time to time with other sera, the histories of most cases being obtained at a later date.

I. COMPARATIVE VALUES OF VARIOUS GONOCOCCUS ANTIGENS

As shown in the tables, about 60 per cent. of all cases reacted positively with one or more of the gonococcus antigens.

Of particular interest are the comparative results with Antigens 1 and 3. The first, Antigen 1, is a simple suspension of gonococci in normal saline solution and Antigen 3, a filtrate of gonococci autolyzed in normal saline solution. Antigen 1 yielded 58.9 per cent. positive reactions, while Antigen 3 yielded 47.9 per cent. In 51.1 per cent. of cases the reactions with Antigen 1 were stronger than those with Antigen 3, in 39.5 per cent. the reactions were equal in both, and in 9.3 per cent. Antigen 1 yielded weaker reactions.

Antigen 4 yielded but 18.7 per cent. positive reactions. We used this antigen in an amount equaling one-fourth its anticomplementary dose, with 0.1 to 0.2 c.c. of serum.

Antigen 5, an alcoholic extract of gonococci, yielded but 9.3 per cent. positive reactions, and in all of these the reactions were weak. This supports the general observation that alcohol does not serve to extract the antigenic principle of bacteria and is unsuitable for bac-

terial antigens, however desirable it may be from the standpoint of stability.

The filtrates of gonococcus Antigens 2 and 4 were used in testing a number of sera and yielded a much smaller percentage of positive reactions. These antigens were difficult to titrate because of a tendency to become hemolytic. The degree of complement-fixation in positive reactions was always slight, and the readings were difficult.

It is seen that best results were secured with a simple antigen composed of gonococci suspended in sterile normal saline solution plus a preservative. Even in normal saline solution, autolysis rapidly occurs but it appears that the bacterial protein, aside from the endotoxins, possesses antigenic principles and they add to the antigenic value of the preparation. Of great importance in the preparation of gonococcus antigen is the use of as many different strains as possible. The difficulty of isolating cultures of gonococci and keeping them under continuous cultivation over a long period of time and the ever present chances of having the cultures contaminated are factors adding greatly to technical difficulties in the preparation of gonococcus antigens.

II. MIXED INFECTION IN CHRONIC GONOCOCCUS INFECTIONS

All of the cultures of streptococci, staphylococci and pseudodiphtheria bacillus were isolated from cases of chronic urethritis and prostatitis.

By preparing antigens of these organisms, an effort was made to determine their activity in these chronic processes according to whether or not bacteriolytic amboceptors were present in the sera of such cases, in so far as these could be demonstrated by complement-fixation experiments.

In seven, or 9.6 per cent. of cases the antigen of streptococci yielded positive reactions. Four of these were cases of chronic urethritis and prostatitis and three were well marked cases of pyosalpingitis.

In five, or 6.8 per cent. of cases the antigen of white staphylococci yielded positive reactions. One of these was a case of chronic urethritis and prostatitis and four were of pyosalpingitis.

In eight cases, or 11 per cent. the antigen of cultures of staphylococcus aureus yielded positive reactions. Four of these were cases of chronic urethritis and four of pyosalpingitis.

TABLE 1
ANTICOMPLEMENTARY TITRATION OF ANTIGENS

Dose of Antigen	Gonoc. 1 1:50	Gonoc. 2 1:200	Gonoc. 3 1:20	Alcohol. Ext. Gonoc. 1:50	Streptoc. 1:200	S. Albus 1:200	S. Aureus 1:200	Pseudod. B. 1:200	M. Catar- rhalls 1:200	Mening. C. 1:200	Mixed 1:200
0.4 c.c.	H.	H.	H.	H.	H.	H.	H.	H.	H.	H.	H.
0.8 c.c.	S. I. H.	H.	M. I. H.	H.	H.	H.	H.	H.	H.	H.	H.
1.0 c.c.	M. I. H.	H.	I. H.	H.	S. I. H.	H.	H.	H.	H.	H.	H.
2.0 c.c.	I. H.	S. I. H.	I. H.	S. I. H.	M. I. H.	S. I. H.	S. I. H.	S. I. H.	S. I. H.	S. I. H.	S. I. H.
Antigenic dose used	0.2 c.c.	0.5 c.c.	0.2 c.c.	0.5 c.c.	0.4 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.

H = Complete hemolysis.

S. I. H. = Slight inhibition of hemolysis.

M. I. H. = Marked inhibition of hemolysis.

I. H. = Complete inhibition of hemolysis.

TABLE 2.
COMPLEMENT-FIXATION IN GONOCOCCUS INFECTION OF MALES.

No.	Gonoc. 1	Gonoc. 2	Gonoc. 3	Gonoc. 4	Alcoh. Exl. Gonoc.	Streptoc.	S. Albus	S. Aureus	Pseudod. b.	M. Catarrhalis	Meningoc.	Mixed	Diagnosis
1	++	++	++	0	0	0	0	0	0	0	0	0	Chronic urethritis fourteen years
2	++	++	++	0	0	0	0	0	0	0	0	0	Arthritis; no evidence of urethritis.
3	++	++	++	0	0	0	0	0	0	0	0	0	Denies infection. Several years. No
9	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis. No
10	++	++	++	++	0	0	0	0	0	0	0	0	discharge for three months.
11	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Three weeks. Arthritis.
12	++	++	++	++	0	0	0	0	0	0	0	0	Gonorrheal arthritis.
13	++	++	++	++	0	0	0	0	0	0	0	0	Acute urethritis. One week.
14	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Sixteen days.
15	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis.
16	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis. Three years.
17	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Seven weeks.
18	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Six weeks.
19	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Five weeks.
20	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis and prostatitis.
21	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis. Ten years.
22	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis and prostatitis. Seven
23	++	++	++	++	0	0	0	0	0	0	0	0	years.
27	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis.
28	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Third attack.
29	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Second attack.
30	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis.
31	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis. Seventeen years.
32	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis and prostatitis.
42	++	++	++	++	0	0	0	0	0	0	0	0	Urethritis. Five months.
43	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis. Four years.
44	++	++	++	++	0	0	0	0	0	0	0	0	Chronic gonococcus epididymitis.
45	++	++	++	++	0	0	0	0	0	0	0	0	Chronic urethritis and prostatitis.

TABLE 3
COMPLEMENT-FIXATION IN GONOCOCCUS INFECTION OF FEMALES.

	Gonoc. 1	Gonoc. 2	Gonoc. 3	Gonoc. 4	Alcoh. Ext. Gonoc.	Streptoc.	S. Albus	S. Aureus	Pseud. b.	M. Catarrhalis	Meningoc.	Mixed	Diagnosis
13	++	++	++	++	+	+	++	++	+	0	+	++	Bilateral pyosalpingitis.
14	++	++	++	++	+	+	++	++	+	0	+	++	Bilateral pyosalpingitis.
15	++	++	++	++	+	+	++	++	+	0	+	++	Abortion case. Indefinite history.
25	++	++	++	++	+	+	++	++	+	0	+	++	Indefinite history. Probably lues.
26	++	++	++	++	+	+	++	++	+	0	+	++	Bilateral pyosalpingitis.
68	++	++	++	++	+	+	++	++	+	0	+	++	Salpingitis and lues.

TABLE 4
COMPLEMENT-FIXATION IN VAGINITIS OF CHILDREN.

	Gonoc. 1	Gonoc. 2	Gonoc. 3	Gonoc. 4	Alcoh. Ext. Gonoc.	Streptoc.	S. Albus	S. Aureus	Pseud. b.	M. Catarrhalis	Meningoc.	Mixed	Diagnosis
No.													
4	+	+	+	+	0	+	+	+	+	+	+	+	Acute gonorrhoeal vaginitis. One month.
5	+	+	+	+	0	+	+	+	+	+	+	+	Scanty discharge. Negative vaginal and vulvar smears. Slight vaginitis.
6	+	+	+	+	0	+	+	+	+	+	+	+	Acute gonorrhoeal vaginitis. One month.
7	+	+	+	+	0	+	+	+	+	+	+	+	Pus about cervix.
8	+	+	+	+	0	+	+	+	+	+	+	+	Acute vaginitis. No discharge at present.
33	+	+	+	+	0	+	+	+	+	+	+	+	Slight vaginitis. No gonococci at present.
34	+	+	+	+	0	+	+	+	+	+	+	+	Gonorrhoeal vaginitis and ophthalmia.
35	+	+	+	+	0	+	+	+	+	+	+	+	Pus about cervix.
36	+	+	+	+	0	+	+	+	+	+	+	+	Acute gonorrhoeal vaginitis of five weeks' duration.
37	+	+	+	+	0	+	+	+	+	+	+	+	Slight vaginal discharge. No gonococci in discharge.

*For more particulars relative to the clinical aspect of these cases see paper of Dr. John F. Sinclair. Archiv. of Pediatrics, December, 1913.

Five, or 6.8 per cent. of cases yielded positive reactions with the antigen of cultures of a pseudo-diphtheria bacillus. Two of these were cases of chronic urethritis and prostatitis and three of pyosalpingitis.

The filtrates of these antigens, prepared after the method already given, presented the same hemolytic properties as noted with the filtrates of the gonococcus antigens. These filtrate antigens were likewise used with a number of sera but the percentage of positive reactions was much less, and the degree of complement-fixation so slight as to make accurate readings quite difficult.

Thus it was found that in about 9 per cent. of cases, all chronic infections, these mixed organisms were insufficiently active to cause the production of amboceptors. This would indicate their importance as secondary factors in these infections and that they were not leading merely a saprophytic existence. This was found especially true in the cases of pyosalpingitis.

III. PRACTICAL VALUE OF THE GONOCOCCUS COMPLEMENT-FIXATION REACTION

The difficulty of isolating and preserving a sufficient number of cultures of real gonococci in order to prepare a satisfactory polyvalent antigen constitutes a weighty drawback in the practical use of the gonococcus complement-fixation test.

In the majority of reactions the amount of complement-fixation was much less than that commonly occurring in syphilis. Hence the reactions were not so satisfactory as those secured in the majority of syphilis reactions, and the readings were more difficult.

Because of the small amount of gonococcus antibody present in the majority of cases, unless accompanied by unusual complications, the technic of the test and particularly the adjustment of the hemolytic system, required considerable care in order to secure best results.

In a comparative study of a number of sera tested with both the antisheep and antihuman hemolytic systems slightly better results were secured with the latter. In using the antihuman system exactly the same technic was employed as with the antisheep system except that two hemolytic doses of hemolysin were used instead of one. In both, inactivated sera were used. The stronger reactions with the antihuman system showed the probable influence of natural antisheep hemolysin in human sera, when an antisheep system is employed.

In 52.6 per cent. of cases the results were the same with both systems; in three cases, or 15.6 per cent. the reactions were slightly positive and one other was stronger with the antihuman and negative or weaker with the antisheep system; in one case the reaction was negative, and in three others weaker with the antihuman, and positive or stronger with the antisheep system. Thus with the antihuman system slightly better results were secured. This was further supported by a similar set of comparative reactions with sera from which the natural antisheep hemolysin had been removed.

The following table gives a summary of the reactions with the ninety-two sera examined:

TABLE 5.
SUMMARY OF COMPLEMENT-FIXATION REACTIONS IN GONOCOCCUS INFECTIONS.

Diagnosis	Reactions		Per Cent. Positive
	+	-	
Urethritis: Week 1-4	6	7	46.1
Week 4-8	3	2	60.0
Week 8-12	3	2	60.0
Over one year	19	10	65.5
Arthritis of probable gonococcus infection	5	1	83.3
Vaginitis (children)	5	5	50.0
Chr. salpingitis	4	2	66.6
Controls: Normal	0	7	0
Lues.	1	8	11.1
Chaneroids.	0	2	0

(a) Positive reactions in uncomplicated urethritis were uncommon under the fourth week of the infection.

(b) As complications developed and more of the body cells were activated to the production of antibodies, the percentage of positive reactions became greater.

(c) The reaction is of particular value in aiding the diagnosis of the nature of an obscure arthritis; in pelvic inflammatory diseases of women; in the examination of women attendants of children; in the determination of whether or not a given case of urethral infection is cured, or still harbors foci of living gonococci; and in the diagnosis and management of gonorrheal vaginitis of female children. Our percentage of positive reactions in such cases of vaginitis would indicate that the infection ascends higher than the cervix more frequently than is generally supposed.

IV. BIOLOGICAL RELATIONSHIP OF THE GONOCOCCUS

The determination of the biological relationship by complement-fixation tests of a micro-organism to others of a group bearing similar morphological or cultural characteristics depends on whether or not the organism produces a specific amboceptor, and the antigen in the test possesses the distinct antigenic principle.

Immune sera, as antimeningococcus sera, differ widely in their amboceptor content as measured by a complement-fixation technic, and hence not all sera are suitable for such study.

Of main interest in this connection is the possible biological relationship of the gonococcus and meningococcus. From the standpoint of similarity in morphology, the micrococcus catarrhalis is also of interest in this connection.

We have studied these relationships by two main methods:

(a) By interactions of the immune sera of these micro-organisms with the respective antigens, and (b) by testing all sera of gonococcus patients with polyvalent antigens of meningococcus and the micrococcus catarrhalis. The results may be summarized as follows:

1. The gonococcus amboceptor fixed complement best with its own antigen, to a less degree with meningococcus antigen, and not at all with an antigen of catarrhalis.

TABLE 6.
ANTIGONOCOCCUS SERUM.

Dose of Serum	Antigens				
	Gon. 1 0.2 c.c.	Gon. 2 0.5 c.c.	Gon. 3 0.2 c.c.	M. Cat. 0.5 c.c.	Meningo. 0.5 c.c.
.01 c.c.	H.	H.	H.	H.	H.
.05 c.c.	S. I. H.	H.	H.	H.	H.
.1 c.c.	S. I. H.	H.	S. I. H.	H.	H.
.2 c.c.	M. I. H.	S. I. H.	M. I. H.	H.	S. I. H.
.2 c.c.	H.	Control			

TABLE 7.
ANTIMENINGOCOCCUS SERUM.

Dose of Serum	Antigens				
	Gon. 1 0.2 c.c.	Gon. 2 0.5 c.c.	Gon. 3 0.2 c.c.	M. Cat. 0.5 c.c.	Meningo. 0.5 c.c.
.01 c.c.	H.	H.	S. I. H.	H.	H.
.05 c.c.	H.	H.	H.	H.	H.
.1 c.c.	S. I. H.	S. I. H.	S. I. H.	H.	H.
.2 c.c.	S. I. H.	S. I. H.	M. I. H.	H.	S. I. H.
.2 c.c.	H.	Control			

The sera used in the experiments illustrated in Tables 6 and 7 were obtained in the open market. Sera of other make obtained in the same way gave similar results.

2. The meningococcus amboceptor in both sera of different sources, while fixing complement to but a slight degree with meningococcus antigen, reacted equally well with gonococcus antigens; no fixation occurred with catarrhalis antigen.

3. These results indicate the close biological relationship of the gonococcus and meningococcus and while their respective amboceptors were most specific for their own antigen, yet in lower dilutions of serum this specificity was lost and the results constituted an example of "group" reaction as seen in similar studies with the group of streptococci, diphtheria bacilli, etc.

4. As shown in Tables 2, 3 and 4, complement-fixation with sera of gonococcus infections reacted positively with the antigen of meningococcus in 13.8 per cent. of cases. This may be considered further evidence of the close relationship of the amboceptor of these organisms, rather than an indication of the presence of a separate meningococcus antibody.

SUMMARY

About 60 per cent. of all cases of gonococcus infections reacted positively in the gonococcus complement-fixation test. In the few cases of pyosalpingitis examined, 66 per cent. reacted positively. The highest percentage of positive reactions, 83 per cent., occurred in cases of arthritis, considered clinically as possible gonococcus infections.

The gonococcus complement-fixation test is of particular value in aiding the diagnosis of the nature of an obscure arthritis, in pelvic inflammatory diseases of women, to deciding whether or not a given case of urethral infection is cured or still harbors foci of living gonococci and aiding in the diagnosis and management of vaginitis in female children.

The reactions are not generally as satisfactory as those occurring in the syphilis reaction because the quantity of gonococcus antibody is much smaller unless grave and widespread gonococcus metastases exist, and the fixation of complement by bacterial amboceptor and antigen is not so marked as that occurring with syphilis reagin and a lipoidal extract.

In a comparative study of a number of sera tested with both the antishoop and antihuman hemolytic systems slightly better results were secured with the latter.

To be of any value gonococcus antigens must be polyvalent. An antigen composed of a simple suspension of organisms in saline solu-

tion yielded 11 per cent. better reactions than filtrates. It appears that the bacterial protein, aside from the endotoxins, aids in the antigenic effect. Alcoholic extracts of gonococci possess little or no value.

The occurrence of positive reactions in about 9 per cent. of cases of chronic gonococcus infections with antigens of staphylococci, streptococci and diphtheroid bacilli, indicates the active rôle these organisms may assume in these infections. The occurrence of about 5 per cent. positive reactions with an antigen of the micrococcus catarrhalis would indicate that this organism may be likewise active in chronic urethritis.

A study of antigonococcus and antimeningococcus sera with antigens of gonococci and meningococci, indicates the close biological relationship of the gonococcus and meningococcus, and while their respective amboceptors are most specific for their own antigens, in lower dilutions this specificity is not so apparent and the results constitute another example of "group" reaction similar to those occurring with the group of streptococci, diphtheria bacilli, spirochetes, etc.

We wish to express our appreciation of the kindness of Drs. Uhle and McKinney, Dr. John F. Sinclair, Dr. A. W. Bowker, Dr. Berta Meine and Dr. A. J. Casselman for furnishing the sera and clinical histories used in this work.

A SIMPLE METHOD OF CULTIVATING BACILLI, PREFERRING CONDITIONS OF PARTIAL ANAEROBIOSIS (B. ABORTUS, BANG; B. BIFIDUS, TISSIER)

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Nowak's¹ idea, that in the growth of the bacillus subtilis in an enclosed vessel an appreciable amount of oxygen is used, thereby bringing about a condition of partial anaerobiosis which in turn favors the growth of certain organisms, is founded on solid reasoning. In following Nowak's line of thought, numerous methods have been adopted which have met with more or less success. One method that commands attention is the placing of nutrient agar-agar in the bottom of a mason jar, sterilizing and then slanting it. After streaking the surface with the bacillus subtilis, agar slants, previously inoculated with suspected (abortion) material, are placed in the jar, which is then tightly sealed. The jar with its contents is incubated at 37° C. for varying lengths of time.

The one objection to this expedient, however, is that the time required for initial growth varies from three to ten days and in the larger number of cases from seven to ten days. To obviate the necessity of such delay in obtaining the results sought the following method was conceived:

Employ an ordinary large test tube such as is generally used in making potato cultures. In this insert a tightly fitting piece of thin glass which should be about three-fourths the length of the test tube. Place plain agar-agar, as culture medium in the tube and insert a cotton plug. Sterilize the tube thus prepared. After this process slant the tube so that the agar lies high on one side of the central glass slide. Carefully observe the tube and note this, as exactness in this regard has a direct bearing on the success of the technic. The water of condensation will tend to collect at the bottom of the lower side thus minimizing the chance of surface spreading or contamination of the higher side.

1. *Ann. de l'Inst. Pasteur*, 1908, 22, p. 541.

Streak the lower side with the *bacillus subtilis*, taking care to cover the whole surface. Inoculate the higher side with some aborted material, amniotic fluid from an aborted calf, or the fluid from the interior of a cyst of the ovary. After inserting the cotton plug, push it down into the tube and place a tightly fitting rubber cork over it to make the tube air-tight. The tube thus prepared must be incubated at 37° C.

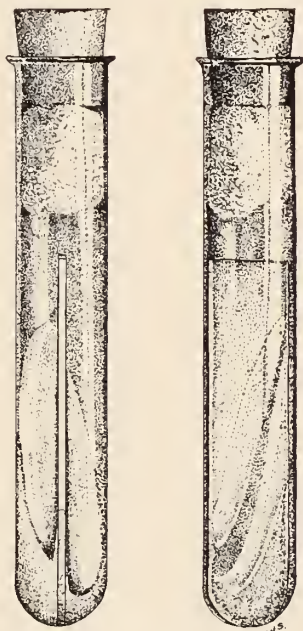


Fig. 1.—Showing glass slide in tube.

Within two to three days growth will take place on the higher side where the suspected material was planted, thus enabling a further study of the organisms or colonies present. The writer has had excellent results in using this method for the cultivation of the *bacillus bifidus*, Tissier, and the *bacillus abortus*, Bang.

To aid in this practice it is suggested that test tubes with the glass slide be made all in one piece, thus precluding all possibility of the *bacillus subtilis* contaminating the desired growths. So constructed, the tubes may be slanted in the usual way, there being no need for having one side of the slant higher than the other.

AN ATTENUATED CULTURE OF TRYPANOSOMA BRUCEI*

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SYNOPSIS

INTRODUCTION.

ATTENUATION OF TRYPANOSOMA LEWISI.

CULTURE OF TRYPANOSOMA BRUCEI.

MORPHOLOGY OF THE CULTURAL TRYPANOSOMES.

EFFECTS ON ANIMALS.

INOCULATION OF RATS; INFLUENCE OF AGE AND CULTURE ON INFECTIVITY;

EFFECT OF SMALL DOSES OF CULTURE.

INOCULATION OF MICE.

INOCULATION OF DOGS.

INOCULATION OF RABBITS.

INOCULATION OF GUINEA-PIGS.

COURSES OF THE INFECTION IN RATS.

SUMMARY.

INTRODUCTION

The production of active immunity may be brought about by the introduction into the animal body of living organisms either fully virulent or attenuated, or dead organisms or their products. The injection of virulent organisms either in sublethal dose, or into parts of the body not used by the organism as a channel of entrance in the natural infection, is uncertain and often dangerous. On the other hand, the injection of dead organisms, though not usually attended with danger, is often ineffectual because with the death of a germ, depending on the method of treatment, the antigen or immunity-producing substance may be partially destroyed. The introduction of a non-virulent or slightly virulent organism would then, *a priori*, suggest itself as the most suitable for the production of immunity.

It may be well to mention that attenuation may be produced in various ways, that is, by treatment of organisms with chemical substances; by exposure to moderately high temperature, or to the action of light; by cultivation in immune serum; by passage through relatively insusceptible animals; and finally, by long-continued cultivation outside of the animal.

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Protection against experimental infection by the various organisms may be obtained by application of one or more methods of immunization referred to. This is particularly true of the bacterial diseases, which for obvious reasons, have claimed the most attention in the past. On the other hand, with very few exceptions, very little has as yet been done in the immunity against protozoal diseases. This has been due chiefly to the fact that pure cultures of the pathogenic protozoa have not been available. With the successful cultivation of certain trypanosomes it has been possible to approach this problem along experimental lines. Among the strictly pathogenic flagellates, the *trypanosoma brucei* has thus far been cultivated *in vitro* for many generations.

The problem which presented itself was to ascertain whether or not the cultures of this protozoan could be rendered avirulent or attenuated, and further, whether such modified cultures would be capable of conferring immunity. This paper deals only with the question of attenuation produced by long continued cultivation of this organism. As will be shown in the following pages, this result has been in part accomplished, for while our modified strain is still somewhat pathogenic for susceptible animals, it has practically lost its pathogenic power for others.

Attenuation of this organism, then, would suggest the possibility of obtaining similar results with the other pathogenic trypanosomes when they are successfully cultivated.

ATTENUATION OF THE *TRYPANOSOMA LEWISI*

The first demonstration of the attenuation of a cultural trypanosome was made by Novy, Perkins and Chambers.¹ The organism studied was *trypanosoma lewisi* of a rat. Their culture isolated in September, 1905, at first produced typical infection in rats. After a long interval the culture was again tested as to its virulence in March, 1909, and after numerous trials it was found that it did not produce infection, and in fact, had become completely attenuated. The organism was at this time in its one hundred and fifty-eighth generation. Since a considerable interval had elapsed, in which the virulence was not tested, it could not be determined whether the loss of virulence was gradual or quite sudden. Neither could it be determined just how early after isolation such loss had occurred.

1. *Jour. Infect. Dis.*, 1912, 11, p. 411.

It was desirable, accordingly, to confirm this work on another strain of *trypanosoma lewisi*, and at the same time to ascertain just how early evidence of attenuation could be obtained. Accordingly a culture of *trypanosoma lewisi* was isolated in October, 1911. This was maintained by weekly transplants on a blood agar medium consisting of equal parts of defibrinated rabbit's blood and nutrient agar. These cultures were grown at a temperature of approximately 25 C. They invariably gave very rich growth, which at times could be seen as whitish colonies not unlike those of bacteria. These cultures produced typical infection in rats for a considerable period after isolation. Evidence of attenuation first appeared in the fifty-first generation, which culture on injection into two rats produced typical infection in one, and only a slight infection of rather short duration in the other (see Table 1).

TABLE 1.
RESULTS OF INOCULATION OF CULTURES OF THE *TRYPANOSOMA LEWISI*.

No. of Rat	Generation of Culture	Period of Incubation Days	Duration of Infection Days	Remarks
2.25	51	2	12	Examination discontinued 12 days after inoculation
3.25	51	2	9	The number of organisms seen was never more than 3 per field
3.118	59	4-5	—	On the ninth day after inoculation when the number of organisms was 25 per field the rat was bled
4.118	59	—	—	No infection
1.67	65	5-6	1	2 organisms seen
2.67	65	6-7	3	$\frac{1}{2}$ per field, 3, 1 organism seen
9.149	69	3-5	1	2 organisms seen
10.149	69	—	—	No infection
3.190	71	—	—	No infection
4.190	71	—	—	No infection
4.104	75	5-6	4	One organism seen
5.104	75	—	—	No infection

A second test, made with the fifty-ninth generation, showed even more marked attenuation. Of two rats inoculated one showed no organisms in its blood, while the number in the other never reached more than twenty-five per field (No. 7 objective).

The third test, with the sixty-fifth generation, gave still better evidence. In one of the two rats inoculated trypanosomes were found only on the sixth day, at which time but two could be detected. In the other rat the parasites appeared on the seventh day, when they were about 0.2 per field. On the eighth day only three and on the ninth only one organism could be found.

A test of the sixty-ninth generation gave in one rat a transient infection, which lasted but one day. Only two organisms were found, while its companion failed to show any parasites.

The inoculation of the seventy-first generation into two rats yielded no infection.

The last test, made with the seventy-fifth generation, gave a negative result in one rat, and a mild infection of very short duration in the other.

In all the tests above mentioned the rats used for the experiment were young, and a natural infection was excluded by daily examination for at least two weeks before they were used in an experiment. In each test a rat was given an intraperitoneal injection of the contents of one cultural tube, which was grown for seven days at 25 C. After the injection the rats were examined as a rule every day for at least two weeks.

While confirming the production of attenuation, the fact is established that the loss of virulence becomes evident in fifteen months, a period represented by sixty or more generations.

CULTURE OF *TRYPANOSOMA BRUCEI*

It is well known that *trypanosoma brucei* was first cultivated by Novy and MacNeal in 1903.² The medium used was defibrinated blood agar similar to that employed in the previous successful cultivation of the *trypanosoma lewisi*.³ The agar was prepared by adding to the meat extract (1-8) 2 per cent. peptone, 0.5 per cent. sodium chlorid, 1 per cent. normal sodium carbonate, and 2 per cent. agar. One part of this agar was mixed with two parts of defibrinated rabbit blood, and the mixture solidified in an inclined position. The medium was inoculated with two drops of defibrinated rat blood, very rich in trypanosomes. At 25 C. growth took place in the water condensation, and no visible colonies could be made out. Of fifty animals thus tested Novy and MacNeal found that only four gave positive results. Smedley⁴ found that three out of ten attempts were positive.

Because of the inconsistent results it seemed advisable to attempt an improvement of the medium. Various attempts were made in this direction. The best results were obtained by the employment of a dialyzed meat extract, which was made as follows: 125 gm. chopped beef, and 250 c.c. of water were allowed to digest over night in the cold, or for one hour at 55 C. The mixture was then strained and the extract boiled and filtered. The filtrate was then dialyzed in a large collodium sac against running distilled water for twenty-four to

2. *Jour. Amer. Med. Assn.*, 1903, 41, p. 1266; *Jour. Infect. Dis.*, 1904, 1, p. 1.

3. *Contributions to Med. Research Dedicated to V. C. Vaughan*, 1903, p. 549.

4. *Jour. Hyg.*, 1905, 5, p. 38.

forty-eight hours. The dialyzed sac contents were diluted to 1 liter with distilled water, and then 2 per cent. peptone, 0.5 per cent. sodium chlorid, .01 per cent. calcium chlorid, 1 per cent. normal sodium carbonate, and 2 per cent. agar added. About 1 c.c. of this agar was placed in each tube and sterilized in an autoclave by heating to 105 to 108 C. for fifteen minutes. Shortly before use the desired number of agar tubes were melted in the water bath, cooled to 60 C., and two volumes of defibrinated rabbit blood were added. The mixture was well agitated, and then allowed to solidify in a slanting position.

A number of experiments were made with a medium in which the meat extract was replaced by an extract made of peas and beans, and obtained by boiling 1 per cent. of each with distilled water. To this extract the usual amounts of sodium chlorid, alkali, and agar were added. On the addition of two parts of defibrinated rabbit blood a medium was obtained which gave more constant results than those obtained with the original meat extract (1 to 8).

A third medium was employed similar to that employed by Nicolle.⁵ In this no meat extract was employed. It was prepared by dissolving 2 per cent. agar, 2 per cent. peptone, and 0.5 per cent. sodium chlorid in distilled water, no alkali being added. It was diluted with two volumes of defibrinated rabbit blood, the same as in the case of the other media.

In order to ascertain the relative value of these media as compared with the original medium of Novy and MacNeal a series of cultures were carried out. For this purpose the blood of an infected rat was transferred to not less than six, usually twelve tubes of each media, and incubated in the usual way at 25 C. Six comparative trials were thus made. The results of these tests were decidedly favorable to the dialyzed meat extract medium, since 80 per cent. of the tubes with this medium gave a positive growth. In the case of the pea and bean medium 53 per cent. of the tubes were positive. The modified medium of Nicolle gave about the same result, namely, 48 per cent. of successful cultures. The least favorable results were obtained with the original medium, since only 25 per cent. of these were successful.

No advantage was found by altering the amount and kind of alkali in these media. It was hoped to secure better results by keeping the inoculated tubes in atmospheres of different gases, such as hydrogen,

5. *Arch. de l'Inst. Pasteur de Tunis*, 1908, p. 55; *Ann. de l'Inst. Pasteur*, 1909, 23, p. 361.

nitrogen and carbon dioxid, but all such attempts proved very unsatisfactory, and we found it best to adhere to the ordinary aerobic conditions.

Having a satisfactory nutrient agar, attempts were made to improve the blood constituent of the medium. For this purpose defibrinated rabbit blood was centrifugated, and the serum drawn off and diluted to the original blood volume with 0.5 per cent. sodium chlorid solution. The clear serum was then mixed with the dialyzed nutrient agar, in a ratio of two to one. The red blood cells, freed from serum, were likewise diluted with salt solution to the original blood volume, and this suspension added to the nutrient agar (2 to 1). On inoculation of these two media with trypanosomal blood it was found that the serum agar gave practically 100 per cent. successful cultures, whereas the medium containing only red blood-cells gave but 38 per cent. Inactivation of the serum for one-half to one hour gave essentially the same results as the diluted serum agar. It would seem advisable, therefore, in attempting isolation of *trypanosoma brucei* to employ dilute serum rather than whole defibrinated blood. We have found that this organism maintains itself without any difficulty on this serum medium, although no hemoglobin is present. In fact the growth on this medium becomes extremely rich, and is easily visible to the eye. For ordinary purposes the blood agar medium has been used since it is more easily prepared.

To obtain an initial culture it is advisable to employ the blood of a rat which is in the early stage of the infection, having from ten to thirty-five trypanosomes per field. The results do not seem to be as good when the inoculation is made with blood obtained from the later stages of the disease, when the parasites are more numerous. In the inoculated tubes the cultural forms of trypanosomes may occasionally be seen as early as the sixth day. They are more likely to appear on the twelfth or fourteenth day, and exceptionally they may be delayed as late as twenty-one days.

After obtaining such excellent results with nagana it would seem that this medium could be used for the cultivation of the other pathogenic trypanosomes. This expectation, however, was not realized, and although many attempts were made to cultivate the trypanosomes of sleeping sickness, caderas, surra, and dourine, they were invariably unsuccessful.

The culture used in the present work was isolated on March 15, 1910, on the pea and bean blood medium. After four generations (weekly transplants) on this medium, it was transplanted to dialyzed nutrient blood agar, and since this gave a richer growth the culture has been maintained from that time on the latter. The blood used in these cultures was drawn from the carotid artery of a rabbit under aseptic conditions, and immediately defibrinated. It was then drawn up into a bulb, and placed in the ice chest for three to four days before use. It was found that such blood was more suitable than blood freshly drawn and immediately used. Furthermore, tubes inoculated immediately after slanting did not give as good a growth as those kept in the ice chest for a day. The transplants were made by means of a pipet instead of the loop used in the transplantation of the *trypanosoma lewisi*.

At present (Jan. 1, 1914), the culture is in its one hundred and ninety-third generation, and shows no indication of degeneration or exhaustion; in fact, it is in a most excellent condition, and invariably yields a very rich growth, which at times is evidenced by a faint whitish film on the surface of the medium. (On July 3 the culture is in the two hundred and sixteenth generation.)

MORPHOLOGY OF THE CULTURAL TRYPANOSOMES

In the cultures the trypanosomes occur either as single, free-swimming cells, or in groups consisting of a varying number of organisms, from five to six to as many hundreds. These groups at times attain great size, filling up the field (No. 7 objective). The groups do not have the regular symmetrical arrangement of the cultural rosettes of the *trypanosoma lewisi*, but present the picture of a disorderly, writhing mass similar to a Medusa head. At times, however, fairly symmetrical rosettes occur that bear a striking resemblance to a "sun-burst." In all these groups the flagella are directed outward. There is a marked variation in the cultural forms, short, stubby spindle forms being found side by side, with long thin ones, the latter preponderating. The organisms often occur in pairs attached at their posterior ends. An undulating membrane, though present, is not in any way conspicuous, as in the blood forms; this is due to the fact that the micro-nucleus, as a rule, is situated anterior to the nucleus.

In cultures grown on the ordinary blood-agar the cells show, instead of a homogeneous cytoplasm, one or two highly refractive globules which may be of considerable size. In the later generations, espe-

cially when grown on the dialyzed blood-agar medium, the globules become smaller and more numerous, and not infrequently cells entirely devoid of these granules have been found. The presence of large intracellular globules may probably be considered as pointing to a somewhat unfavorable medium, and their decrease or absence would indicate an adaptation of the trypanosomes to their environment, or at least a decided improvement in the quality of the medium.

The transplantation of the culture after many generations on blood medium to serum agar gives a good growth which is often heavier than that in blood agar, and is readily seen as a white scum on the surface of the medium. The fact that the organism can be maintained indefinitely on the serum-agar medium would indicate that the presence of hemoglobin is not necessary to the culture of the *trypanosoma brucei*. In this connection it may be of interest to state that transplantation from the blood-agar culture to ascitic-fluid agar was entirely unsuccessful in three separate attempts.

EFFECTS ON ANIMALS

Novy and MacNeal⁶ were the first to try the effect of inoculations of cultures into animals. They found that the survivals in tubes, in which no multiplication had taken place, infected rats and mice on the fifth and even on the ninth or tenth day, although failures to infect were noted in two tests with eight-day material. When the cultures of the organism were obtained these were found to be almost as virulent as the rat strain of the virus. The intraperitoneal injection of virulent cultures (Generations 2-5) was found to produce death in mice and rats at times in three and one-half days, usually in seven or eight days, and exceptionally as late as eleven days. The period of incubation in the case of the animals infected with such cultures varied from three, seven or nine days, but in most cases organisms were first seen on the fifth day after inoculation. Since the intraperitoneal injection of virulent blood usually killed rats and mice in from three to five days, it would seem as if this culture, even in early generations, had already undergone partial attenuation.

The results of Novy and MacNeal's experiment further showed that the age of the culture, temperature at which the tubes were kept and composition of the medium greatly influenced the virulence of the organisms. Tubes incubated at 25 C. and then transferred to 34 C. for

6. *Jour. Amer. Med. Assn.*, 1903, 41, p. 1266; *Jour. Infect. Dis.*, 1904, 1, p. 1.

one or two days quickly lost their virulence for rats and mice. As regards the effect of age, they found that cultures which were 18 to 20 or more days old were usually non-infective. Their views, that cultures when kept for from three or four weeks at 25 C. will lose their virulence, has been confirmed by the results which will be given later.

Smedley⁷ carried the trypanosoma brucei through three generations on the Novy-MacNeal medium. He failed to produce an infection in one rat and three mice, the cultures used being either first, second or third generation. Smedley explains this by stating that the cultures were probably too old, and that possibly the amount of material injected was too small. He injected into the mice one or three loopsful, and into the rat 1 c.c. of the material. The age of the cultures injected was 15, 25, 28 and 32 days. It will be shown later that Smedley's negative results were undoubtedly due to the fact that the cultures were too old.

Our own experiments with cultures have fully confirmed the fact that they are infective for the ordinary laboratory animals. This is particularly true for the early generations. In order to demonstrate the loss of virulence, if any, by prolonged cultivation, it was necessary to make tests at long intervals of time. Inasmuch as there was reason to believe that no material loss of virulence occurred during the first year of cultivation the animal inoculations were not made with any regularity until after that time. Before going into a detailed consideration of these tests, it is desirable to indicate the method employed.

The cultures, unless otherwise indicated, were always grown at 25 to 27 C. for seven days. The tubes employed were 12 to 15 mm. by 150 mm. The medium which has been used almost from the first was prepared with the dialyzed meat extract, already mentioned. Before using a culture for injection, it was examined to ascertain its condition. At the end of seven days the tubes invariably showed an abundance, in fact, an extremely rich growth, consisting of perfectly formed, very actively motile flagellates.

By means of a sterile, drawn-out tube pipet, about 1 c.c. of sterile salt solution (0.85 per cent.) was introduced into the tube and the growth then taken up and transferred to a sterile tube. This suspension was invariably taken up in a sterile syringe and injected intraperitoneally into the animal. In order to avoid any deleterious action of the ordinary distilled water, the salt solution was always made up with glass-redistilled water. Since even this solution might have some

7. *Jour. Hyg.*, 1905, 5, p. 40.

injurious action on the organisms, special care was taken to effect the transfer from the culture tube to the animal in the shortest possible time. As a rule, when only a single culture was injected, this did not require more than two minutes.

INOCULATION OF RATS

The white rat is extremely susceptible to *trypanosoma brucei*, for even a single trypanosome is capable of producing a rapidly fatal infection. The period of incubation, following the intraperitoneal injection of a small dose (0.1 to 0.01 c.c.) of the blood is very short, the parasite appearing in the blood of the inoculated rat within twenty-four, or at most forty-eight hours, and death occurs on the fourth or fifth day. The rat is, therefore, an excellent reagent for testing the virulence of the cultured trypanosome, and for that reason has been utilized more than any other animal in our work.

The inoculation of the first generation of the *trypanosoma brucei* gives variable results, depending largely on the age of the material. It may be assumed that the organism in this first generation goes through a sort of a transition stage, while accommodating itself to its new environment. The new cultural form which develops from the blood type is necessarily not as abundant as in the subsequent generations. If such a culture is injected in the early stage of its production it may possibly contain survivals of the original blood forms, and the infection may be due to such. On the other hand, if it is injected after three weeks, the cultural forms present may become so enfeebled by the prolonged action of temperature and medium as to be incapable of producing an infection. On examination of Table 2 it will be seen that all the rats (eleven) inoculated with the first generation, between the fifteenth and the fortieth day of cultivation, survived, and at no time showed flagellates in their blood. Of the thirteen rats inoculated with the first generation on the sixth to the eleventh day, nine became infected after a period of incubation of from four to six and eight days, and died on the eighth to the sixteenth day, whereas four were negative.

The second and subsequent generations do not show the variations noted in connection with the first generation, as the trypanosome has adapted itself to the new living conditions. The cultures develop promptly and are invariably rich at the end of seven days. The injection of a single tube of such a culture in the second generation, as

TABLE 2.
INOCULATION OF RATS WITH CULTURES OF *TRYPANOSOMA BRUCEI* GROWN AT 25 C.*

No. of Rat	Culture		Period of Incubation, Days	Death, Days	No. of Rat	Culture		Period of Incubation, Days	Death, Days
	Generation	Age, Day				Generation	Age, Days		
1	1	6	6-8	12†	57	92	7	4-7	42
2	1	10	4-6	8	58	92	7	4-7	24
3	1	10		—	59	93	7	4-7	13
4	1	10		—	60	93	7	4-7	12
5	1	10	6-8	16	61	130	7	6-7	22
6	1	10	6-8	16	62	130	7	6-7	20
7	1	11	4-6	8	63	131	7	3-6	16
8	1	11	4-6	14	64	131	7	3-6	16
9	1	11	4-6	8	65	136	7	2-6	31
10	1	11	4-6	14	66	136	7	2-6	53
11	1	11	4-6	14	67	137	7	2-6	40
12	1	11		—	68	137	7	2-6	28
13	1	11		—	69	138	7	2-6	31
14	1	15		—	70	138	7	2-6	64
15	1	20		—	71	139	7	6	30
16	1	20		—	72	139	7	6	27
17	1	20		—	73	140	7	6	30
18	1	21		—	74	140	7	6	30
19	1	21		—	75	144	7	6-7	29
20	1	28		—	76	144	7	8-9	28
21	1	29		—	77	144	7	6-7	26
22	1	32		—	78	150	7	5-7	69
23	1	40		—	79	150	7	9-11	60
24	1	40		—	80	152	7	6-8	8
25	2	7	3-4	7	81	152	7	6-8	38
26	2	7	5-6	9	82	153	7	5-7	8
27	2	7	6-7	10	83	153	7	5-7	60
28	2	7	5-6	9	84	154	7	8-10	126
29	2	7	4	9	85	154	7	8	¶
30	32	7	4-5	8	86	154	7	8	18
31	40	7	12-13	21	87	159	7	12-13	32
32	42	8†	14-16	29	88	159	7	23-24	52
33	50	7	6-7	12	89	160	7	7-8	¶
34	50	7	5-6	10	90	160	7	8-9	73
35	57	7	8-9	12	91	160	7	8-9	¶
36	61	7	5-6	10	92	164	7	8-9	57
37	61	7	6-7	12	93	164	7	6-8	28
38	75	8	5-8	25	94	166	7	7-9	18
39	75	8	11-12	26	95	166	7	7-9	18
40	75	8	8-10	28	96	166	7	6-8	34
41	80	7	4-5	22	97	167	7	19-21	53
42	84	7	5-7	26	98	168	7		68
43	84	7	5-7	40	99	168	7	12-14	¶
44	85	7	6	41	100	168	7	25-27	35
45	85	7	6	39	101	168	7	15-17	29
46	86	7	7	43	102	169	7		35
47	86	7	7	48	103	169	7		—
48	87	7	7	46	104	169	7	18-20	50
49	87	7	7	39	105	169	7	26-28	39
50	88	7	3-8	42	106	170	7	21-24	55
51	88	7	3-8	44	107	180	7	9-12	62
52	89	7	6	30	108	180	7	7-9	25
53	89	7	6	94	109	181	7	6-7	83
54	90	7	7-11	21	110	190	7	6-7	78
55	90	7	7-11	21	111	190	7	6-7	—
56	91	7	7-11	21	—	—	—	—	—

* In the above table each rat received the culture present in one tube, except Nos. 5, 6, and 13, which received each 4 cultures, and Nos. 10, 11 and 12, where 2 cultures were given each.

† This culture was grown for seven days at 25°, then placed for one day at 33° C.

‡ Blood survivals were present.

§ Death was not recorded.

¶ No infection occurred.

¶ These rats were bled for experiments on the fifteenth and sixteenth days.

shown in Table 2, gives a prompt infection, the parasites appearing in the blood in from four to seven days, and death results on about the fourth day after the trypanosomes have once been found. The short duration of the disease indicates that the organism is quite as virulent as that of the original blood.

After the tests with the second generation, none were made for some time, but were resumed with the thirty-second generation. The rat (Rat 30) inoculated with this culture gave the usually short period of incubation, but, unfortunately, a record of its death was not made. The serial tests made with cultures up to the seventy-fifth generation (Table 2) show, with the exception of Rats 31 and 32, that the duration of the infection from the time of inoculation (ten to twelve days) was not materially different from that produced by the second generation. The long survival (twenty-nine days) of Rat 32 must be ascribed to the exposure of the culture to 33 C. for one day. As shown by Novy and MacNeal, cultures at this temperature become non-virulent in two or more days. The course of the infection in Rat 31 was not due to this cause, but probably to a faulty inoculation.

After cultivation for one and a half years, that is, with the seventy-fifth generation, the duration of the infection became definitely prolonged, indicating a decided change in the infectivity of the trypanosomes. Rats 38, 39 and 40 inoculated at this time (Table 2) died on the twenty-fifth, twenty-sixth and twenty-eighth day, respectively. An inspection of Tables 2, 3 and 4 will show that the inoculation of seven-day cultures from the seventy-fifth to the one hundred and ninetieth generation, with very few exceptions, yields a chronic infection (Chart 1) which lasts more than twenty-five days, and in one instance was even prolonged to 126 days. The few instances of death in less than twenty days are often due to some other cause, since the number of trypanosomes in the blood, in such cases, is usually too small to produce a fatal result. In only four instances (Rats 98, 102, 103, Table 2; Rat 16, Table 3) did the rats fail to become infected. The uniformly positive results on inoculation has led us to believe that either these rats were accidentally not inoculated, or else the injections were made into the intestines. When Rat 16 (Table 3) was reinoculated sixty-three days later with a seven-day culture (Generation 100) it showed trypanosomes in its blood on the sixth day and died on the eighteenth day.

The average duration of the infection, consequent on inoculation of a seven-day culture, beginning with the seventy-fifth generation, was found for sixty-two rats listed in Table 2 (Rats 38 to 108, inclusive, less nine of uncertain duration), to be thirty-eight days, whereas the average with earlier generations for the twelve rats (Rats 25 to 37, Table 2) is but twelve and one-half days. This fact clearly shows that the culture has undergone considerable modification since the first year of cultivation, and it justifies the belief that in time the seven-day culture will become wholly avirulent for rats.

TABLE 3
SHOWING THE VIRULENCE OF CULTURES WHEN KEPT FOR 7, 14, 21 AND 28 DAYS

Ages of Cultures												
Generation	7 Days			14 Days			21 Days			28 Days		
	No. of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days	Death, Days	No of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days	Death, Days
84	1	5-7	26	21	6	28	41	—	—	61	—	—
84	2	5-7	40	22	6-13	25	42	—	—	62	—	—
85	3	6	41	23	7	84	43	8-11	51	63	—	—
85	4	6	39	24	7-18	23	44	8-12	32	64	—	—
86	5	7	43	25	8-11	39	45	—	—	65	—	—
86	6	7	48	26	8-11	37	46	8-18	21	66	—	—
87	7	7	46	27	—	—	47	—	—	67	—	—
87	8	7	39	28	—	—	48	—	—	68	—	—
88	9	3-8	42	29	—	—	49	—	—	69	—	—
88	10	3-8	44	30	—	—	50	—	—	70	—	—
89	11	6	30	31	11-14	37	51	—	—	71	—	—
89	12	6	94	32	—	—	52	—	—	72	—	—
90	13	7-11	40	33	—	—	53	—	—	73	—	—
90	14	7-11	21	34	—	—	54	—	—	74	—	—
91	15	7-11	21	35	7-11	19	55	13-17	41	75	—	—
91	16	0*	—	36	4-7	29	56	—	—	76	—	—
92	17	4-7	42	37	7-13	30	57	—	—	77	—	—
92	18	4-7	24	38	4-7	11	58	6-8	32	78	—	—
93	19	4-7	13	39	—	—	59	—	—	79	—	—
93	20	4-7	12	40	—	—	60	11-14	265	80	—	—
Average		7.15	37.50		10-7	33		13.3	73.7			
Percentage of infection			100			55			30			0

* No infection.

The period of incubation, when a seven-day culture is used, does not vary as much as the duration of the infection. As a rule, daily examinations of the blood were not made. The rats were examined more often every two or three days, and so an exact period of incubation was not established. The average of the figures given in Tables 3

and 4 will be found to be about six days, though this is probably higher than it would be if daily examinations had been made. When this is compared with the five and four-tenths' day average inoculation of the five rats which received the second generation (Table 2), it will be seen that no material difference exists in this regard between the earliest and latest generations.

INFLUENCE OF AGE OF CULTURE ON INFECTIVITY

It has been pointed out in connection with Table 2 that the first generation of a culture, 15 to 40 days old, failed to infect rats. Novy and MacNeal⁸ obtained similar results, and concluded that it was very probable that the cultures, when kept for three or four weeks at 25 C., lose their virulence. Inasmuch as no experiments had been made to test this point, it seemed desirable to ascertain just what influence age exerted on the pathogenicity of a culture. For this purpose a series of tests were made with ten consecutive generations, beginning with the eighty-fourth.

Since prolonged exposure of a culture to 25 C. causes considerable change in the medium and consequently in the form, and even destroys the life of the organism, it was found best to transfer the cultures, after they had been developed at 25 C. for seven days, to a cool room, the temperature of which, while not constant, was usually 10 to 15 C. Under these conditions the cultures showed little or no change during the first two weeks, but by the end of the third week, when actually 28 days old, considerable alteration of form was noted. A goodly number of the organisms, however, were still actively motile, and when transplanted to a fresh medium invariably gave a growth, which, though slight, on further transplantation yielded typical rich cultures and these infected rats the same as the original seven-day culture. It may be noted, in this connection, that the 28-day-old culture, while transplantable, was without effect on animals (Table 3).

At the end of seven, fourteen, twenty-one and twenty-eight days, after inoculation, the cultures were tested on rats in the usual way. The rats were injected in duplicate, each receiving the contents of one tube. The results of this series of tests are summarized in Table 3. It will be seen from this table that the cultures, when grown for seven days at 25 C., produced infection in nineteen of the twenty rats tested, the single exception being that of Rat 16. The possible reason

8. *Jour. Amer. Med. Assn.*, 1903, 41, p. 1266; *Jour. Infect. Dis.*, 1904, 1, p. 1.

for this failure has already been given, and it is safe to conclude, in the light of all the other positive results obtained with seven-day cultures (Table 2), that a culture at this stage of its growth is invariably infective to rats.

On the other hand, the fourteen-day cultures (seven days at 25 C., and seven days at room temperature) were found to infect only eleven out of twenty rats, that is, 55 per cent. The twenty-one-day cultures were even less infective, only six out of twenty (33 per cent.) being positive, whereas the twenty-eight-day cultures were uniformly non-virulent. It will be seen, therefore that the increase in age of the

TABLE 4
SHOWING THE VIRULENCE OF CULTURES WHEN KEPT FOR 7, 14 AND 21 DAYS

Ages of Cultures									
Generation	7 Days			14 Days			21 Days		
	No. of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days	Death, Days	No. of Rat	Period of Incubation, Days,	Death, Days,
136	1	2-6	31	11	—*	—	21	—	—
136	2	2-6	53	12	—	—	22	—	—
137	3	2-6	40	13	—	—	23	—	—
137	4	2-6	28	14	—	—	24	—	—
138	5	2-6	31	15	6-9	39	25	—	—
138	6	2-6	64	16	6-9	64	26	—	—
139	7	6	30	17	2-6	60	27	—	—
139	8	6	27	18	2-6	46	28	—	—
140	9	6	30	19	2-9	12	29	—	—
140	10	6	30	20	2-9	33	30	—	—
Average			6	36.4		8	42.3		
Percentage of infection			100			60			0

* No infection, consequently no death occurred.

cultures (Generation 84-93) is accompanied by a rapid loss of virulence, the infection dropping from 100 per cent. at the end of seven days to 55 per cent. in fourteen days, 33 per cent. in twenty-one days, to *nil* per cent. in twenty-eight days.

After an interval of one year the cultures were again tested and the results brought together in Table 4. Since the twenty-eight-day cultures in the previous trial had been found to be non-infective, no tests were made with tubes of this age. The cultures were now in the one hundred and thirty-sixth to one hundred and fortieth generation, and

were, therefore, nearly three years out of the animal body. As before, the seven-day culture proved to be infective for all the rats (ten) tested, or 100 per cent.; the fourteen-day culture infected six out of ten rats, or 60 per cent., thus showing practically no change. The twenty-one-day culture, however, failed to infect, thereby indicating a change in the organisms as compared with the previous year.

It would not be safe to conclude from the foregoing that the twenty-one-day cultures were always devoid of pathogenicity. Thus, when rats were given from five to ten bi-weekly injections of such a culture, with the object of immunization, five out of twenty-one (23 per cent.)

TABLE 5
SHOWING EFFECT OF MULTIPLE INJECTIONS OF 21 DAY OLD CULTURES

No. of Rats	Generation	No. Injections	Period of Incubation Days	Death, Days	Remarks
1	144-147	6	—	—	No infection
2	144-147	6	—	—	No infection
3	144-147	6	—	—	No infection
4	144-147	6	—	—	No infection
5	144-147	6	—	—	No infection
6	144-147	6	—	—	No infection
7	144-146	5	—	16	No infection
8	144-147	6	—	—	No infection
9	144-146	5	3-18	41
10	144-147	6	—	—	No infection
11	144-146	5	4-15	53
12	144-146	5	10-15	46
13	144-146	5	12	48
14	144-146	5	—	15	No infection
15	144-148	9	—	32	No infection
16	144-148	9	30	142
17	144-149	10	—	—	No infection
18	144-149	10	—	—	No infection
19	144-149	10	—	—	No infection
20	146-151	10	—	—	No infection
21	146-151	10	—	—	No infection

The period of incubation is not definitely determined; the figures given represent the last negative and first positive examination.

became infected (Table 5). It would seem, therefore, that variations in the conditions of cultivation, such as slight differences in the medium or in the rate of multiplication, influence appreciably the pathogenicity of the twenty-one-day cultures. It is quite possible that in a given tube a culture may rapidly develop and as a result undergo degeneration more readily and thus become non-infective, while a companion tube with less growth may contain virulent organisms. This would seem to be the reason why frequently only one or two rats became infected when inoculated with apparently the same material. The set of twenty-

one-day rats which received multiple inoculation were given identically the same cultures, and yet only 23 per cent. developed trypanosomes. This variation can hardly be explained as due to individual susceptibility of the rats.

The period of incubation, after injection of a culture, was not determined as accurately as perhaps it should have been. It is to be regretted that daily examinations of the blood for trypanosomes were not made, but this was quite impossible owing to the large number of animals which required attention. As shown in Tables 2 and 4, the rats which received the seven-day culture usually showed trypanosomes on the sixth or seventh day, the average period of incubation in the former table being seven and in the latter six days. Had daily examinations been made, it is not unlikely that the average would have been reduced to nearly five days. The average period of incubation, when the fourteen-day culture was used, is apparently longer, for in Table 3 it comes to ten days, while in Table 4 it is eight days. In the case of the twenty-one-day culture the average is increased to thirteen days. It is rather to be expected that with the progressive weakening of the culture, the incubation period should be prolonged.

The duration of the disease, as expressed in Tables 2-4, refers to the number of days which elapsed between the date of inoculation and of death. It would seem as if this could be given with accuracy, but such is not quite the case, for the reason that, not infrequently, the test animal succumbs to an intervening disease. Thus Rats 19, 20 and 38 (Table 3) died in thirteen, twelve and eleven days, respectively. Neither at the time of death nor at any previous day did they show more than a few parasites, and this small number could not account for the rapidly fatal outcome. Exceptionally, when the animal is in an enfeebled condition, trypanosomes may appear in large numbers (about 100 per field) on about the fourteenth day, and death may be due to this cause. In general, however, the infection persists for several weeks, at times for two or three months; and in one instance (Rat 60, Table 3) it lasted for 265 days.

The average duration for rats inoculated with the seven-day culture is 37.5 days (Table 3) and 36.4 days (Table 4); for those which received the fourteen-day culture the averages are 33 and 42.3 days, respectively; while for those which were given the twenty-one-day culture the average is 73.7 days. This latter high average is due to the extremely long survival of Rat 60, and if this is excluded the average drops to 35.4 days.

It will be seen, therefore that the average duration of the infection in rats produced by the *trypanosoma brucei* when cultivated for two or three years is approximately the same, although considerable individual variation, as seen in the tables, does exist.

EFFECT OF SMALL DOSES OF CULTURE

It will be seen from the foregoing results that one tube of a seven-day culture, even in the latest generations, invariably infects the white rat, and that such cultures when kept for an additional period of from one, two or three weeks, progressively lose their infecting power. If the latter fact was merely due to a decrease in the number of organisms, then a somewhat similar result could be expected by injecting fractional doses of a single culture.

With the object of determining the least infective dose, a seven-day culture was taken up in sterile salt solution (0.85 per cent.) and diluted in series so as to yield 1:10, 1:100, 1:1,000, etc., suspensions. When 1 c.c. of these suspensions was injected intraperitoneally into each of two rats, it was found that the 1:1,000 suspension uniformly failed to infect. Thus, eighteen rats, in sets of two, received such suspensions from nine different cultures, and in none was an infection obtained.

The tests made with the 1:10 and 1:100 dilutions gave frequent, though by no means constant infections. In the early summer of 1912 eighteen rats, most of which had received previous injections of fourteen-, twenty-one- and twenty-eight-day cultures, were tested for a possible immunity, each receiving one-tenth of a culture tube, and every one became infected. A few months later, in October, four consecutive tests, made on eight clean rats, gave a negative result with the one-tenth-culture dose, and it seemed therefore as if the organism had become considerably less virulent during the few intervening months. Further tests, however, showed that this avirulence was merely temporary, due to some as yet unrecognized cause.

In endeavoring to find the cause of this temporary loss of virulence of the one-tenth-culture dose, attention was first drawn to a variation in the procedure of making the subcultures. It was found that the non-infective cultures were prepared by transferring the growth, by means of a pipet, from one tube to each of two fresh blood-agar tubes, whereas in the case of the infecting cultures, the inoculation was made into four or five fresh tubes. It was conceivable, therefore, that the

former received a heavier inoculation, and consequently developed an earlier maximum growth than the latter. As a result of the more rapid growth, an earlier degeneration could be expected, in which case the culture, though but seven days old when used, would be less infective than the more slowly developing cultures. To test this hypothesis, two series of cultures reproducing the above conditions were carried through four generations, and then tested on rats. These cultures (Generation 140) at the end of seven days were equally rich and showed no difference in the appearance of the organisms. The results, given in Table 6, likewise show no difference between the two series as regards the effects on rats. The early death of five out of eight rats was due to an intervening infection. Hence some other explanation had to be sought.

TABLE 6*
SHOWING THE EFFECT ON VIRULENCE OF THE QUANTITY OF CULTURE TRANSPLANTED

Transplants Made from 1 to 2 Tubes				Transplants Made from 1 to 4 Tubes			
No. of Rat	Amount Injected	Period of Incubation, Days	Death, Days	No. of Rat	Amount Injected	Period of Incubation, Days	Death, Days
1	0.1	4-6	28	5	0.1	6-8	43
2	0.1	4-6	7	6	0.1	8-10	14
3	0.01	8-10	43	7	0.01	8-10	14
4	0.01	..	10	8	0.01	10-13	14

* Generation 140 was used in this experiment.

Another explanation of the variable results following the injection of one-tenth of a culture was sought in the temperature of the salt solution employed in the preparation of the suspensions. It seemed likely that suspensions made with a moderately warm solution would be less active or even avirulent as compared with those prepared at a lower temperature. Accordingly, a series of comparative tests were made to determine this point. In one set, after the seven-day culture was cooled in ice water, the growth was taken up in iced salt solution (0.85 per cent.) and the desired suspensions were prepared with the chilled solution. Even the syringes employed were first rinsed in the cold, sterile water. For the other set the suspensions were prepared with a salt solution previously warmed to 30 C.

The results obtained in two such tests (Table 7) were so markedly opposed as to make it clear that the temperature of the suspension was not a factor.

A third attempt to account for the above-mentioned results gave an equally unsatisfactory answer. It was suggested that the cultures which usually were kept on the floor of the hot room were possibly exposed to a varying temperature, and that a rise of a few degrees might render the culture more virulent. To bring out the effect of temperature, accordingly a series of tests were made, with cultures grown for seven and nine days, respectively, at 25 to 27 C., and compared with similar tests of cultures grown at 30 C. The nine-day cultures at 30 C., while very rich, showed a considerable number of rounded-up organisms, which, however, were still actively motile, whereas those grown for a like period at 25 to 27 C. contained perfectly normal flagellates.

TABLE 7
SHOWING RESULTS OF INOCULATION WITH SUSPENSIONS MADE AT DIFFERENT TEMPERATURES

Cultures Taken Up in Water at									
0 C.					30 C.				
Generation	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	Generation	No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days
149	1	0.1	†	—	149	5	0.1	6-8	38
149	2	0.1	†	35	149	6	0.1	10-12	26
149	3	0.01	†	16	149	7	0.01	6-8	26
149	4	0.01	†	—	149	8	0.01	†	—
150	9	0.1	15-18	39	150	13	0.1	†	—
150	10	0.1	8-12	29	150	14	0.1	†	—
150	11	0.01	†	—	150	15	0.01	†	—
150	12	0.01	†	—	150	16	0.01	†	—

† No infection occurred.

Where no death is recorded, the rats lived for more than 5 months uninfected.

On examination of Table 8 it will be seen that the injection of one-tenth of a culture produced an infection in fifteen out of twenty-four rats, while that of one-hundredth of a culture gave a positive result in only eight, or one-third of the rats tested. The death of a number of the rats within two weeks after inoculation was due to a troublesome intercurrent infection, and undoubtedly affected the total result. On the whole, however, it is safe to conclude that neither the temperature nor duration of cultivation influenced the virulence of the culture.

Therefore, the occasional failure of a fraction of a culture to infect is not due to any one of the three factors discussed above, neither can it be due to a variation in the medium, since the same nutrient agar was employed in all tests. Only one explanation seems to remain,

TABLE 8
SHOWING THE EFFECT OF TIME AND TEMPERATURE ON THE VIRULENCE OF CULTURES

Cultures Grown at 25-27 C. for						Cultures Grown at 30 C. for					
7 Days						7 Days					
No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	9 Days		No. of Rat	Amount Injected, Tube	Period of Incubation, Days	Death, Days	9 Days	
				No. of Rat	Amount Injected, Tube					No. of Rat	Amount Injected, Tube
Exp. 1											
Gen-eration 1	0.1	8-10	41	5	0.1	6-8	13	8-10	69	13	0.1
2	0.1	8-10	58	6	0.1	†	10	12-14	59	14	0.1
3	0.01	†	—	7	0.01	6-8	11	†	26	15	0.01
4	0.01	†	—	8	0.01	†	12	14-16	*	16	0.01
Exp. 2											
Gen-eration 1	0.1	5-7	22	21	0.1	†	25	3-5	52	29	0.1
18	0.1	5-7	33	22	0.1	†	26	7-9	36	30	0.1
19	0.01	11-13	60	23	0.01	7-9	27	†	14	31	0.01
153	0.01	5-7	64	24	0.01	11-13	28	†	6	32	0.01
Exp. 3											
Gen-eration 33	0.1	†	19	37	0.1	†	41	†	—	45	0.1
34	0.1	9-11	17	38	0.1	†	42	†	23	46	0.1
35	0.01	†	10	39	0.01	†	43	†	10	47	0.01
36	0.01	†	—	40	0.01	†	44	†	—	48	0.01
153											
Gen-eration 153	0.1	8-10	43	9	0.1	8-10	9	8-10	69	13	0.1
	0.1	8-10	42	10	0.1	8-10	10	8-10	59	14	0.1
	*	10-12	*	11	0.01	†	11	†	26	15	0.01
	6-8	6-8	*	12	0.01	†	12	14-16	*	16	0.01
	11-13	11-13	9	25	0.1	†	25	3-5	52	29	0.1
	7-9	7-9	8	26	0.1	†	26	7-9	36	30	0.1
	†	†	36	27	0.01	†	27	†	14	31	0.01
	†	†	20	28	0.01	11-13	28	†	6	32	0.01
	8-10	8-10	—	41	0.1	†	41	†	—	45	0.1
	†	†	9	42	0.1	†	42	†	23	46	0.1
	†	†	5	43	0.01	†	43	†	10	47	0.01
	†	†	—	44	0.01	†	44	†	—	48	0.01

*Death was not recorded. †See foot-notes to Table 7.

namely, a variation in the number of trypanosomes injected. Previous to making up a suspension the cultures were always examined, and it is possible that in some instances an unusually rich mass of flagellates was thus removed from the tube, thus decreasing the number actually injected. The injection of a definite number of cultural trypanosomes, ascertained by direct count, would probably throw light on this question.

INOCULATION OF MICE

As expected mice were found to be very susceptible. The period of incubation, following the intraperitoneal injection of a seven day culture, was about the same as in rats, and varied somewhat with the amount injected. Thus, as shown in Table 9, it was about four to six

TABLE 9
SHOWING THE RESULTS OF INOCULATION OF MICE WITH CULTURES

Generation	No. of Mice	Amount Injected Tube	Period of Inoculation, Days	Death, Days
143	1	1	5-6	18
143	2	1	†	2
143	3	0.1	†	—
143	4	0.1	5-6	13
143	5	0.01	7-8	17
143	6	0.01	†	
144	7	1	4-6	9
144	8	1	4-6	15
144	9	0.1	6-8	12
144	10	0.1	6-8	23
144	11	0.01	8-10	14
144	12	0.01	8-10	12
145	13	0.1	6-8	14
145	14	0.1	6-8	14
145	15	0.01	8-12	14
145	16	0.01	8-12	14
145	17	0.001	8-12	*
145	18	0.001	8-12	13

* Death was not recorded. † See foot-notes Table 7.

days when an entire culture was injected; with one-tenth and one-hundredth of a culture it was prolonged to six to eight and eight to ten days, respectively. As will be seen, even one-thousandth of a culture is capable of producing an infection. One of the mice which received this small dose (Mouse 17) showed trypanosomes in its blood on one day, and although examined repeatedly during the next few months they were not found again. While this would seem to be a recovery, it is more probable that an error in observation was made. In the routine examination of a large number of animals it has been

customary to make eight to ten or more fresh blood preparations on a slide, and then examine these in regular order. Notwithstanding the care taken to avoid mistakes in making such serial examination they occurred at times. The duration of the disease, from the time of inoculation, was less than observed with the rats; averaging fourteen and four-tenths days with nine days as the minimum, and twenty-three days as the maximum.

INOCULATION OF DOGS

Only a limited number of tests with dogs were made, and these sufficed to show that the cultures, approximately three years under cultivation, were infective. The injection was made, as usual, intraperitoneally. Dog 1 received five injections, each of ten tubes, in the course of sixteen days. Parasites appeared in its blood on the

TABLE 10
SHOWING THE RESULTS OF INOCULATION OF CULTURES INTO DOGS

No. of Dog	Weight, gm.	Date of Inoculation	Generation	Amount Injected, Tubes	Period of Incubation, Days	Death, Days
1	8700	Dec. 24, 1912	140 141 142	5x10	13-18	44
2	2950	Jan. 28, 1913	144	10	8	127
3	2900	Feb. 18, 1913	147	1	7-10	19
4	2600	Feb. 18, 1913	147	1/10	No infection	39

eighteenth day, rapidly increased in numbers, and death occurred in forty-four days. Dog 4, which received only one-tenth of a culture, although examined twice a week, failed to show trypanosomes and died on the thirty-ninth day. Dog 3 after receiving an injection of a single culture showed one trypanosome on the tenth day, after which the number rose to ten per field or more. The animal became very sick and died of acute infection on the nineteenth day. On the other hand, in Dog 2, which received an injection of ten cultures, the infection pursued a very chronic course. The parasites appeared on the eighth day and continued to be present, with frequent intermissions, up to the time of death (127 days). The number of trypanosomes was usually only one or two per field, but once it rose to ten per field.

INOCULATION OF RABBITS

The rabbit, when inoculated with the virulent trypanosoma brucei, presents a sub-acute infection characterized usually by a period of incubation of two to six or eight days, though it may be considerably

longer (even thirty-one and forty-nine days) depending on the amount of the virus injected. The duration of the disease is from ten to fifty days,⁹ but with minute doses this may be greatly prolonged.

In view of the fact that the rabbit possesses a fair degree of natural resistance no tests of the infectivity of the culture were made with small amounts of such material. Instead the rabbits in one set were given an intraperitoneal injection of the contents of ten cultures. As shown in Table 11, the rabbits thus injected were found, with one exception, to resist this large inoculation. Rabbit 5 died in thirteen days, but not from trypanosome infection. The other animals,

TABLE 11
SHOWING THE EFFECT OF SINGLE AND MULTIPLE INJECTIONS OF CULTURES INTO RABBITS

No. of Rabbit	Generation of Culture	Date of Injection	Amount Injected, Tubes	Period of Incubation Days	Death, Days	Remarks
1	144	Jan. 28, 1913	10	†		Used for an immunity test on the ninety-eighth day
2	145	Jan. 31, 1913	10	†		—ditto on the ninety-fifth day
3	145	Jan. 31, 1913	10	†		—ditto on the one-hundred and twenty-second day
4	145	Feb. 11, 1913	10	†		—ditto on the one-hundred and eleventh day
5	146	Feb. 11, 1913	10	†	13	
6	147	Feb. 14, 1913	10	†		—ditto on the two-hundred and third day
7	148	Feb. 15, 1913	10	66-70		Alive Jan. 1, 1914 (308 days)
8	145-149	Jan. 31-Mar. 4	10x10	†		Used for immunity test on the ninety-fifth day
9	145-149	Jan. 31-Mar. 4	10x10	17-49	58	Alive July 3, 1914 (518 days)
10	145-147	Jan. 31-Feb. 4	4x10	1-12		Used for immunity test on the one-hundred and tenth day
11	146-151	Feb. 11-Mar. 14	10x10	†		—ditto on the two-hundred and fifth day
12	146-151	Feb. 11-Mar. 14	10x10	†		—ditto on the two-hundred and fifth day
13	146-151	Feb. 11-Mar. 14	10x10	†		—ditto on the two-hundred and fifth day

† No infection occurred.

although examined at least twice a week, failed to show any organisms except in the case of Rabbit 7, in the blood of which a single trypanosome was found on the seventieth day. About 5 drops of blood from the ear of this rabbit were injected into each of two rats. Trypanosomes appeared in the blood of these rats on the fifth day. Ten days later another parasite was detected.

At the same time as the above, six rabbits (Rabbits 8 to 13) were given bi-weekly injections, each of ten tubes, intraperitoneally. Rabbit 10 showed trypanosomes (one per field) on February 12, the twelfth

9. Laveran and Mesnil, *Trypanosomes et Trypanosomiasés*, 1912, p. 440.

day after the first inoculation. Two days later they were slightly increased (four per field), after which they disappeared and were not found again until March 11, 21 and 28, when a single trypanosome was detected on each of those days. Examinations made during the months of April and May did not reveal the presence of the parasite. On June 18 one organism was found; alopecia, ulceration of genitalia, and of nose were noted. After July and August, during which no organisms were seen, further examinations were omitted. At present (July 3, 1914) the animal is in excellent condition, showing no signs of lesions.

Rabbit 9 showed a single trypanosome on March 21, the seventeenth day following the last or tenth injection, (forty-ninth day from the date of the first injection). It was negative on March 25, but on March 28 the trypanosomes were ten per field. It died two days later, the fifty-eighth day from the first injection. The other four rabbits which received multiple injections, showed no indication of an infection until the time when they were used for immunity tests.

It will be seen from these experiments with rabbits that at the end of three years of cultivation, the *trypanosoma brucei* has suffered a marked loss of pathogenicity. Owing to the richness of the material injected the occasional infection must be ascribed to individual susceptibility.

INOCULATION OF GUINEA-PIGS

The inoculation of the virulent *trypanosoma brucei* into guinea-pigs gives rise, as in the case of rabbits, to a subacute or even chronic infection having a period of incubation of two to four days, and of variable duration of fifteen to thirty days,¹⁰ although much longer survivals are known. In view of this fact it is to be expected that the injection of a culture, particularly when attenuated under the artificial conditions of cultivation, should be followed by a strictly chronic infection or none at all. The results of numerous tests show that this actually does happen, and that at the end of three years' cultivation the organism is practically avirulent for the guinea-pig.

It had been shown in this laboratory that the injection of early generations of *trypanosoma brucei* infected guinea-pigs. In our own work we found this to hold true for the fortieth generation, a single culture of which, as shown in Table 13, produced a fatal though somewhat prolonged infection.

10. Laveran and Mesnil, p. 441.

No further test was made with cultures until the one hundred and fifth generation was reached. It was then found that a single culture, given intraperitoneally, failed to infect, and this fact was soon confirmed by a series of similar inoculations. Twenty-five guinea-pigs were injected with the one hundred and fifth to the one hundred and forty-fifth generation, inclusive, each with the culture present in a single tube. Of these, four died within the first week, two in the

TABLE 12

SHOWING NEGATIVE RESULTS AFTER INOCULATION OF GUINEA-PIGS WITH ONE CULTURE.

Generation	No. of Guinea-Pigs Inoculated	Results
105	2	One died on the thirteenth, the other on the twenty-fifth day; both negative
109	2	Examined for 79 days, negative
116	2	Examined for 36 days, negative
131	1	Died on sixteenth day, negative
132	2	One died on twentieth day, other examined for 114 days, both negative
133	1	Died on sixteenth day, negative
134	3	Examined for 130, 136, 154 days, respectively; all negative
135	1	Examined for 156 days, negative
136	1	Died on forty-first day, negative
142	2	Examined for 41, and 95 days, respectively, negative
145	4	One died on the eighth day, another on eighteenth day; the others were examined for 46, 52 days, respectively, negative

TABLE 13

SUMMARY OF POSITIVE RESULTS OBTAINED IN GUINEA-PIGS BY INOCULATION OF CULTURES

No. of Guinea-Pig	Generation	No. of Tubes Injected	Period of Incubation, Days	Death, Days
16.94	40	1	14-15	59
4.190	140	10	39-40	50
5.190	140	10	21-24	210
5.147	146	10	14-18	53
6.147	146	10	28-32	158
4.68	133-139	10x1	51-92	268
5.105	136-144	10x10	7-70	104

second week, four in the third week and one in the fourth week, without showing any trypanosomes. The remaining fourteen animals were under observation for thirty-six to 156 days, after which they were used for immunity tests. At no time could parasites be detected in their blood. It is clear, therefore, that this strain had become attenuated to such an extent that a dose of a single culture was unable to infect.

A summary of these tests, including the four animals that died in the first week is given in Table 12.

Inoculation with Ten Cultures.—Inasmuch as it was evident that a single culture would not infect, it was desirable to test the effect of a larger dose. For this purpose the growth present in ten culture tubes was taken up in the usual way and injected. This was made intraperitoneally except in four guinea-pigs, where the injection was subcutaneous. The examinations of the blood were made twice a week at first, and later on alternate days. The animals were thus kept under observation as long as they lived or until used for immunity tests two to five months later.

Of 72 animals thus inoculated with Generations 133 to 153, inclusive, 5 died within fourteen days and were negative. Of the remaining 67 animals, 2 died during the second, and 7 during the third fortnight, all being negative. Of these 67 animals, 4 became infected after a period of incubation of eighteen, twenty-four, thirty-two and forty days, respectively. In other words, 1 out of every 17 guinea-pigs which survived the first fortnight became infected. It is evident therefore, that the culture when given in large doses is not wholly avirulent for these animals.

This result may be due to particularly rich cultures which happened to be used in those instances, or it may be ascribed to individual susceptibility of the animals. An effort was made to ascertain the cause of this occasional infectiveness by growing the cultures in parallel sets at 25 and 30 C. for seven and nine days, at the same time that similar tests were made with rats (Table 8). Although thirty-four animals were thus tested not a single infection was induced. Likewise tests to ascertain the effect of the temperature of the salt solution which was used to make up the suspension, employing this either ice-cold or at 30 C. failed to show any difference, and the animals, fifteen in number, did not become infected. On the supposition that in the first two positive cases mentioned some of the cultures may have been introduced subcutaneously by accident and that injection by this latter route might be a factor, it was deemed best to make some comparative trials. In one such test two guinea-pigs received the usual intraperitoneal injections, while two others were given subcutaneously a like dose from cultures grown side by side with those used for the former. The latter failed to become infected, while both of the animals which received the intraperitoneal injection became positive.

It is worthy of note that the four positive infections occurred in sets of two, and this fact would offset the explanation of individual susceptibility. The first set received Generation 140 and the second set Generation 146. In view of the failure to trace these results to any definite cause it is reasonable to suppose that some special quality of the blood agar medium, very accidental in its occurrence, influenced the cultures and was responsible for the result. That the result was not due to a mere numerical richness of the culture is seen from the following tests:

Inoculation with One Hundred Cultures.—If the occasional infection of guinea-pigs with a culture were due to a special richness of the material injected it would seem that a much larger dose than that of ten tubes should produce positive results. Accordingly, it was decided to test the effect of one hundred such cultures on each of several animals. The growth from one hundred tubes, which examination showed to be extremely rich, was taken up in 20 c.c. of salt solution, and this suspension was injected intraperitoneally into a guinea-pig. Six animals were given this dose of one hundred cultures. The first and second pairs received Generation 141, the third pair Generation 142. Two of these died within a week and were negative; the remaining four were examined at first daily and later on alternate days for seventy-eight to eighty-four days without revealing any trypanosomes. It is seen, therefore, that while Generation 140 infected two guinea-pigs in a dose of ten tubes, in this case Generations 141 and 142 failed to produce a like result, although the amount injected was tenfold.

Multiple Injections of One Culture.—As a further test of the action of cultures, and especially with the object of securing material for immunity tests, a series of thirteen guinea-pigs were inoculated, either weekly or bi-weekly, each time with the growth of a single culture tube. Six of these died a day or two after having received two, three, four, four, eight and eight injections, respectively, representing an interval of eight to thirty-seven days after the first injection. In these the usual examinations were made with negative results.

Seven of the above set were carried through the tenth and last injection. One died on the sixty-ninth day, and five were used for immunity tests on the ninety-eighth to the one hundred and third day after the first inoculation. At no time did these six show any sign of trypanosomal infection. One of the set, however (Guinea-Pig 4.68, Table 13), which received in the course of forty-one days ten injections of Generations 133 to 139, inclusive, showed a very few trypanosomes on the

fifty-first day after the last injection (ninety-two days after the first). The period of incubation in this case was therefore at least fifty-one days; it may have been longer since obviously it could not be determined which one of the ten injections actually infected. It is interesting to note that although much of the same culture material was used in the other animals, only this one became infected. The course of the infection in this particular guinea-pig will be discussed later.

Multiple Injections of Ten Cultures.—At about the same time as the above, each of another set of twelve guinea-pigs was given bi-weekly injections of ten cultures. Two of the animals died after receiving two and three injections respectively. The remaining ten guinea-pigs were given ten injections each. Of these, two died on the fourteenth and twenty-ninth days after receiving the last injection (45 and 61 days after the first), the examinations of the blood being negative. Seven of the remaining gave likewise negative results on frequent, two or more, weekly examinations, and were therefore used for immunity tests after 30, 42, 55, 55, 70, 70 and 91 days elapsed from the time of the last injections, or 61 to 122 days from the first. One guinea-pig in this set (Guinea-Pig 5.105, Table 13) showed a very slight infection on the seventh day following the last injection (70 days after the first). The period of incubation, as in the other infection with multiple injections (Guinea-Pig 4.68) obviously could not be determined. The course of the infection, which was very slight, will be described later.

Course of the Infection.—The several positive infections on inoculation of cultures which have been referred to in the preceding text are summarized in Table 13, and it will be pertinent to briefly outline the course of the disease, as indicated by the trypanosome contents of the blood in these animals.

Guinea-pig 16.94 received an injection of 1 culture, Generation 40, on January 4, 1911. Daily examinations were made and trypanosomes found in very scanty numbers (0.05 per field) on January 19. After that date the examinations were made two or three times a week until death, which occurred on the fifty-ninth day. The infection was characterized by the continuous presence of trypanosomes in the blood, as seen from the following:

Jan. 14.....	Inoculated	Feb. 7.....	5 per field
" 19.....	0.05 per field	" 9.....	35 " "
" 22.....	0.5 " "	" 14.....	50 " "
" 24.....	0.05 " "	" 17.....	7 " "
" 26.....	1 " "	" 20.....	1/2 " "
" 28.....	.3 " "	" 25.....	2 " "
Feb. 2.....	.3 " "	March 1.....	50 " "
" 4.....	.4 " "	" 4.....	died, 59th day.

This constant presence of parasites, their gradual rise in number to a maximum, then a remission followed by a second rise shortly before death are features fairly characteristic of infection with the virulent *trypanosoma brucei*. Two years later, in only one of the other positive infections was a like course observed, and hence this can be next considered.

Guinea-pig 4.190 received an injection of 10 cultures, Generation 140, on Dec. 24, 1912. Through oversight examinations were made only once or twice weekly during the first four weeks (seven tests); after that, however, they were made daily and parasites were found, after eleven consecutive negative examinations, on February 2. They continued present, as shown in the subjoined tabulation, until death, which occurred ten days later. The rapid course of the infection in this animal is in striking contrast with that of its mate, Guinea-pig 5.190, which received a like injection on the same day.

Dec. 24.....	Inoculated	Feb. 6.....	5 per field
" 2.....	1 per field	" 8.....	25 " "
" 3.....	1 " "	" 9.....	10 " "
" 4.....	5 " "	" 11.....	50 " "
" 5.....	4 " "	" 12.....	died 50th day.

Guinea-pig 5.147 was given an injection of ten cultures, Generation 146, on February 7. Five negative examinations were made during the first fortnight. Parasites were found on February 25, and in increased numbers on the day following; they then disappeared, as shown by negative findings in the next seven examinations. On March 10 and 12 they reappeared in small numbers, but were again absent in the next five tests. On March 24 they appeared for the third time, followed by a considerable increase on the 26th, but on the 28th they were again absent and remained so until death, which occurred on April 1 (fifty-three days).

Feb. 7.....	Inoculated	Mch. 12.....	$\frac{1}{2}$ per field
" 25.....	1 per field	" 24.....	5 " "
" 26.....	10 " "	" 26.....	75 " "
Mch. 10.....	$\frac{1}{2}$ " "	April 1.....	died, 53d day.

The noteworthy feature of the infection in this animal is the occurrence of three remissions, each being characterized by an apparently complete disappearance. Similar intermissions were observed in the remaining guinea-pigs. No sub-inoculation of the blood during these intermissions was made.

Guinea-pig 6.147, the mate of the preceding, received on the same day a like dose. Nine examinations during the following four weeks were negative. Trypanosomes appeared on March 11, were present the next day, and then disappeared. After six negative examinations, on alternate days, they were again found on March 26 and 28, and April 1. Absent on the 8th, they reappeared on the 11th, and again disappeared and remained undetected in the nine following examinations. On May 23 they were again found, but not on the 27th and 30th. The parasites were met with on June 3d and 6th; they were not found in the next ten examinations, which were made between June 10 and July 12.

Feb. 7.....	Inoculated	April 4.....	1 per field
March 11.....	$\frac{1}{2}$ per field	" 11.....	20 " "
" 12.....	$\frac{1}{2}$ " "	May 23.....	2 found
" 26.....	2 found	June 3.....	$\frac{1}{2}$ per field
" 28.....	10 per field	" 6.....	2 " "
		July 15.....	died, 158 days.

Guinea-pig 5.190, the mate of one of the preceding, was given an injection of ten cultures on Dec. 24, 1912. After four negative examinations, trypanosomes were found on January 17. Unlike the rapid course of the disease in its mate, the infection in this animal took on a very chronic course characterized by the frequent presence of parasites, although these occurred only in scanty numbers, as seen from the observations noted below.

Dec. 24.....	Inoculated	Feb. 18.....	3 found
Jan. 17.....	1/20 per field	Feb. 19.....	1 per field
Jan. 18.....	1 " "	Feb. 20.....	1 " "
Jan. 19.....	5 " "	Feb. 21.....	1 " "
Jan. 20.....	1 " "	March 22.....	1 found
Jan. 21.....	1/5 " "	April 8.....	5 per field
Jan. 23.....	1/10 " "	April 11.....	5 " "
Jan. 24.....	1/2 " "	April 25.....	1 " "
Feb. 2.....	1/2 " "	May 6.....	10 " "
Feb. 3.....	1/2 " "	May 13.....	2 " "
Feb. 5.....	1/2 " "	May 16.....	1 " "
Feb. 6.....	1/2 " "	May 30.....	1 " "
Feb. 8.....	1 " "	June 13.....	5 " "
Feb. 9.....	1 " "	July 1.....	2 " "
Feb. 12.....	2 found	July 12.....	3 " "
Feb. 13.....	1 " "	July 15.....	10 " "
Feb. 16.....	2 " "	July 22.....	died, 210 days.

The intermittent presence of trypanosomes, in relatively small numbers, for a long period of time is a marked feature of the infection. The first intermission occurred after January 24, when eight consecutive daily examinations were negative. The second intermission occurred after February 21, when eighteen consecutive examinations failed to reveal the parasite. After March 22, when a single trypanosome was noted, five following examinations were negative. Of four examinations made in the two weeks following April 11, only the last was positive. In the next three weeks, negative findings were made on April 29, May 2 and 9. The organism was seen on the thirteenth and sixteenth, when again a period of remission followed on the twentieth, twenty-third and twenty-seventh of May. On the thirtieth the parasite was found in small numbers (one per field). Ten negative and four positive examinations were made between May 30 and July 15. The number of trypanosomes seen was never more than ten per field. Death occurred on the two hundred and tenth day after the first injection.

A few drops of the ear blood of this animal were injected on January 17 into a white rat and a guinea-pig. Two days later a similar injection was made into another rat. Parasites appeared in the blood of the first rat on the third day, after which they rapidly increased in numbers, reaching 200 per field on February

4. Death occurred on February 11, the twenty-fifth day. In the second rat, a similar rapid infection was noted. The parasites appeared in the blood on the second day, and rose to 200 per field before death, which took place on February 6, the eighteenth day.

In striking contrast, however, the guinea-pig which was inoculated on January 17 failed to show any organism in the first twelve examinations. Parasites appeared on the eighteenth day, February 4, about one-half per field. Six examinations between this date and March 4 were negative. On that date, trypanosomes were present, twenty-five per field. The blood was searched on the 7th, 11th, 14th and 18th, with negative results. The parasites again appeared on March 21, about fifty per field, but four days later they were again absent, and the blood remained free of organisms until May 27, although eighteen examinations were made. The animal was therefore apparently free from organisms for more than two months. Three days later the organisms disappeared and were again absent for a period of eighty-seven days, although nineteen examinations were made, when positive, two trypanosomes were seen; four days later, three were found. August 29, or 224 days from the time of its injection, was the last record of examination. No note of its death was made. On May 26, as a test for the possible presence of trypanosomes, some of the ear-blood of the animal was injected into a rat. Parasites appeared on the thirteenth day (June 8), after which they rapidly increased to 100 per field and then slowly decreased in numbers until after a period of thirty-four days, when they disappeared; in the next seven examinations, which terminated August 28, the animal revealed no parasites. Further examinations and a record of its death were not made.

Guinea-pig 4.68, which received ten injections, each of one culture, in the interval between November 6 and December 17, was the only one of seven similarly treated animals which became infected. Although examined twice a week, it showed no parasites until February 6 (fifty-one days from the last injection), when three trypanosomes were found. After thirty-five negative examinations they were again observed, about one-half per field, sixty-one days later, April 8. Three days later they increased to ten per field, after which they disappeared. Twelve examinations made between that date and May 27 were negative. On May 30 one organism was seen. An examination made four days later was negative, while one made June 6 showed parasites, about one-half per field. Fourteen examinations made between June 6 and July 29 were negative. On the latter date beginning lesions on the genitalia were noticed. August 1 (268 days after the first injection) the animal died in a condition of cachexia and infected with cocci, but no trypanosomes were seen. It will be seen that during this time trypanosomes were observed only five times.

Dec. 17.....	Last Inoc.	April 11.....	10 per field
Feb. 6.....	Two found	May 30.....	1 found
April 8.....	1/2 per field	June 6.....	1/2 per field
		Aug. 1.....	died, 268 days.

Guinea-pig 5.105 was the only one to become infected, out of a set of ten which received ten injections each of ten cultures. These were given in the interval between November 26 and January 28. Seven days later, on February 4, a single trypanosome was observed, previous bi-weekly examinations being negative. The following thirty-four examinations likewise failed to reveal the parasite, which, however, was detected forty-six days later, on March 22 (about one-half per field). The blood was again negative two days later, but on March 26 and 28, and April 1 a few organisms were found. After two negative exam-

inations they were again found on the 11th, but were again missed on two subsequent occasions. Death occurred on April 19, or eighty-one days after the last injection, or 104 days after the first.

Jan. 28.....	Last Inoc.	March 28.....	One per field
Feb. 4.....	One found	April 1.....	Three found
March 22.....	1/2 per field	April 11.....	One per field
March 26.....	One found	April 19.....	died, 104 days.

The course of the infection in the last two guinea-pigs, when contrasted with that observed in the four animals which received only single injections, will be seen to be very mild and characterized by an infrequent occurrence of parasites. This peculiarity is undoubtedly due to the treatment with multiple injections as a result of which a mild degree of immunity was probably established. The remarkably long period in which no trypanosomes could be found suggests that the parasites localize and multiply in some other place than the blood stream, only occasionally appearing in the blood. That this is true in regard to rabbits and guinea-pigs will be shown later. It must be remembered, however, that parasites might be present in the blood in such small numbers as to make their detection by the ordinary examination of a fresh preparation quite impossible. Inoculation of the blood into the very susceptible rat would probably reveal the presence of an occasional trypanosome.

COURSE OF THE INFECTION IN RATS

The injection of seven-day cultures into rats as shown in Table 2 produces a fatal infection which, however, has a considerably longer duration than that produced by the virulent strain. The course may be subacute or chronic, the latter extending to 126 days. The injection of a twenty-one-day culture in one rat gave an unusually long duration of 265 days. As a rule, however, survival beyond fifty days is rather exceptional as will be seen from a study of the tables.

In the subacute type, the trypanosomes gradually increase in numbers until they are about fifty per field. The parasites are constantly present, though occasional remissions occur. In the more chronic cases the organisms at times apparently disappear from the circulation and the blood examinations may remain negative for a week or more. Thus in the rat which resisted 265 days, during a period of three months, the parasites were found only four times and then in very scanty numbers.

The long duration of the disease in rats is not due to the inhibitive action of antibodies, as might be inferred, but rather to the feebleness

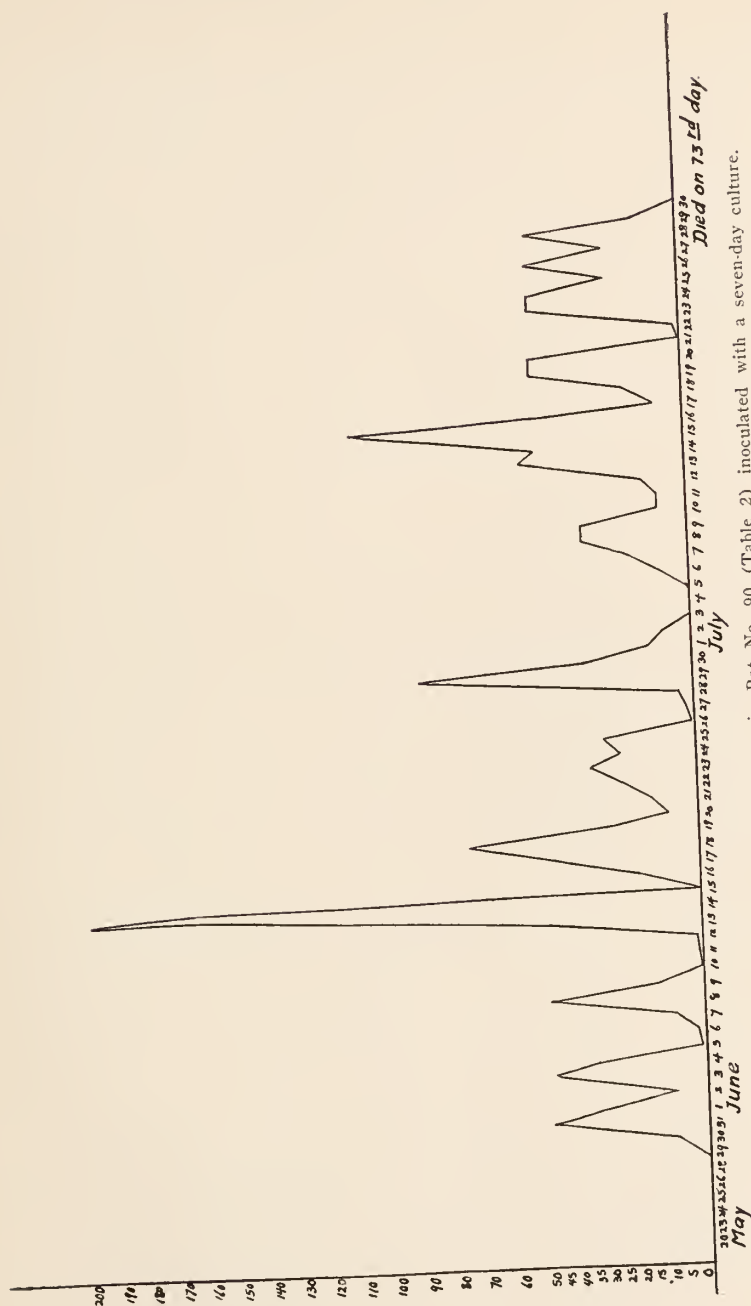


CHART 1.—Variation of number of trypanosomes in Rat No. 90 (Table 2) inoculated with a seven-day culture. The ordinates give number of trypanosomes per field of No. 7 objective while the abscissae show the dates of examination of the blood.

Died on 75th day.

or degree of attenuation of the organisms. The more attenuated an organism is, the longer it will take to restore its pathogenicity. At or shortly before death the trypanosomes have nearly regained their virulence, for on subinoculation, at such time, an acute infection is

TABLE 14

SHOWING RESULTS OF SUB-INOCULATIONS FROM EARLY AND LATE RAT INFECTION INTO RATS.
Early Infection. Started 11 days after Inoculation of Rat 3.22 with Culture

No. of Passage	Date of Inoculation	No. of Rat	Period of Incubation, Days	Death, Days
1	Jan. 20	1.49*	—2	128
1	Jan. 20	2.49	—2	48
2	Jan. 23	3.49*	—3	36
2	Jan. 23	4.49	—3	24
3	Jan. 26	2.60*	—3	46
3	Jan. 26	3.60	—3	9
4	Jan. 29	5.49*	—2	25
4	Jan. 29	6.49	—2	9
5	Feb. 8	8.49*	—4	19
5	Feb. 8	9.49	—4	16
6	Feb. 14	5.98*	—2	39
6	Feb. 14	6.98	—2	25
7	Feb. 27	4.117*	—2	20
7	Feb. 27	5.117	—2	17
8	March 4	3.124	—2	21
8	March 4	4.124*	—2	27
9	March 13	5.141*	—2	12
9	March 13	6.141	—2	13
10	March 15	5.149*	—3	20
10	March 15	6.149	—3	11
11	March 21	5.164*	—4	11
11	March 21	6.164	—4	21
12	March 28	4.177*	—4	10
12	March 28	5.177	—4	9
13	April 6	3.190*	—2	14
13	April 6	4.190	—2	9
14	April 11	5.4	—4	11
14	April 11	6.4*	—4	10
15	April 20	5.19*	—2	5
15	April 20	6.19	—2	5
16	April 25	6.29		3
16	April 25	7.29		3

Late Infection. Started 35 Days after Inoculation of Rat 3.22; 5 Days before Death

1	Feb. 13	4.92*	—3	9
1	Feb. 13	5.92	—3	9
2	Feb. 16	7.99*	—3	6
2	Feb. 16	8.99	—3	8
3	Feb. 22	1.108*	—4	5
3	Feb. 22	2.108	—4	5
4	Feb. 26	7.113	—2	4
4	Feb. 26	8.113	—2	5

* Sub-inoculations were made to the next following pair of rats from the animal marked

produced which by two or three consecutive passages completely restores the virulence so that death results in three to five days. Thus, Rat 1.49 (Table 14) was subinoculated on the day of its death into two rats which died in six and five days, respectively, and a second

passage into two rats reduced the duration to four days. Again, when a subinoculation was made from the same rat two days before death, the animal died in twelve days, and inoculation from the latter produced death in three and four days. On the other hand, subinoculations made from this original rat on the one hundred and second day, that is, twenty-six days before death, caused death in forty-five and ninety-seven days, respectively.

It will be seen from the foregoing that the trypanosomes which develop in the rat after injection of a culture remain attenuated for a considerable period, and that the final result is due to a rather sudden restoration of virulence. As further evidence of this fact reference is made to Table 14, which reproduces the results obtained by making subinoculations at the beginning and shortly before the end of the infection. Rat 3.22, which was used for this experiment, was injected with a seven-day culture on January 9. Trypanosomes appeared on the eleventh day (ten per field) and its blood was inoculated into the first set of two rats, 1.49 and 2.49. A series of successive passages, sixteen in number and extending over ninety-six days, were necessary to restore the virulence to a maximum. Had the subinoculations been made regularly, as soon as trypanosomes appeared in the blood, that is, every two or three days, it is probable that the attenuation of the strain would have been maintained for a much longer period. The second series of subinoculations was started five days before the death of the original rat, which occurred on the fortieth day (February 18) and three or four passages sufficed to restore the virulence.

Of special interest as indicating the attenuated character of the trypanosome present in the rat, after infection with a culture, is the result obtained by injecting guinea-pigs with blood drawn early and late in the course of the infection. Thus, Rat 5.72 received an injection of one seven-day culture, Generation 143, on January 17 and after a period of incubation of six to seven days trypanosomes appeared, three per field. Two drops of this blood, drawn from the tail, was injected into each of two guinea-pigs (Set A), Table 15. On February 13, the day before the death of the rat which occurred on the twenty-eighth day, two drops of its blood (thirty-five per field) were again injected into each of two guinea-pigs (Set B). As seen from Table 15, three guinea-pigs and probably four became infected after a very long period of incubation. Guinea-Pig 4.87, after a period of

incubation of forty-two to forty-six days, showed only a single trypanosome in the fresh blood preparation. Eleven subsequent bi-weekly examinations were negative, but two days before death the organism reappeared (two per field).

Examinations of Guinea-Pig 3.87 never revealed the parasite, but owing to their scarcity probably they were missed. Inasmuch as typical local lesions about the genitalia developed, this seems to have been the case and in all probability the animal became infected. At present (July 3, 1914) the guinea-pig is in excellent condition showing apparently healed lesions.

Guinea-Pig 5 and 6.161 (Set B, Table 15), after frequent examinations for a period of eight months, failed to show either a blood infection or skin lesions. Observations were then discontinued until about ten months after inoculation, when examination showed ulceration and alopecia around the genitalia. Trypanosomes were demon-

TABLE 15
SHOWING THE RESULTS OF SUB-INOCULATION FROM EARLY AND LATE RAT INFECTION INTO
GUINEA-PIGS

Set	No. of Guinea-Pigs	Date of Inoculation	Period of Incubation Days	Results
A	3.87	Jan 24	Apparently no infection; shows healed genital lesion. Alive 524 days (July 3, 1914).
B	4.87	Jan 24	42-46	Died on ninetieth day.
	5.161	Feb. 13	205-266	Posterior alopecia and ulceration of the genitalia. Died in about 300 days.
	6.161	Feb. 13	268-270	Posterior alopecia and ulceration of the genitalia. Died on 404th day.

strated in these lesions. One organism was found in the blood of Guinea-Pig 5.161 on one occasion (two hundredth and sixty-sixth day). Four days later a similar observation was made on Guinea-Pig 6.161.

Guinea-Pig 5.161 died in December. Guinea-Pig 6.161 died on March 24, or 404 days after inoculation.

As shown, heretofore, only a very small number of guinea-pigs when injected with ten cultures, become infected. The greater insusceptibility of these animals is shown therefore in their behavior not only to cultures but also to the blood trypanosomes derived from such cultures.

Reference was made to the possibility of some localization of organisms, suggested by the long periods over which no organisms could be found in the blood stream. Cutaneous localizations of trypanosomes have been observed in the case of rabbits and guinea-pigs.

This condition seems to depend both on the natural resistance of the animal, and on the degree of virulence of the infecting organism. Thus, skin lesions have been produced in rabbits by inoculation of cultures, that is, very attenuated organisms and also of virulent trypanosomes. It is well known that rabbits have a relatively higher resistance to blood infections than guinea-pigs.

In case of guinea-pigs, no skin lesions, so far as we have observed, have resulted from inoculation of virulent organisms. On the other hand, an attenuated strain may be produced by inoculation of cultures into rats and when such organisms are inoculated into guinea-pigs, skin infection frequently results. As shown, these infections are characterized by very long duration and by the appearance of only occasional organisms in the blood.

Having shown that prolonged cultivation of the *trypanosoma brucei* results in an attenuation of the organism, which fact is seen either in a failure of such cultures to infect, or in an increased duration of the disease, it was of interest to determine the least infecting dose of virulent trypanosomal blood and compare this with that of the attenuated organism present in rat blood. Inasmuch as the results of this work will appear in another paper it will be sufficient at present to state that while the minimum infecting dose of the normal virulent strain for rats is represented by a single organism, at least three of the attenuated trypanosomes are necessary for infection. In guinea-pigs, one normal trypanosome is sufficient to infect, while the attenuated strain, as just shown, yields very inconstant results, when many hundreds or even thousands of organisms are injected.

SUMMARY

Trypanosoma lewisi gradually loses its virulence on cultivation, and is practically non-infective in the seventy-fifth generation.

A dialyzed nutrient agar, plus serum (1 to 2) yields the most constant results in isolation of *trypanosoma brucei*. The *trypanosoma brucei* is capable of growing on hemoglobin-free medium.

The continued cultivation of *trypanosoma brucei* markedly attenuates the parasite, the latter becoming avirulent, except in few cases,

for guinea-pigs and rabbits, and less virulent for rats, mice and dogs. In time, probably a wholly avirulent strain can be obtained.

A single culture, seven days old, as yet invariably infects the rats, but when kept at room temperature for an additional one, two or three weeks it becomes less infective or not at all. Multiple injections of such aged cultures in some cases produce infection. The non-virulent cultures, twenty-eight days old, are still capable of growth in vitro.

Cultures induce a subacute or chronic infection, the longest survival in rats being 265 days; in guinea-pigs, 268 days.

The blood of rats infected with cultures is infective for guinea-pigs after a very long period of incubation, and the guinea-pigs develop marked local lesions, especially about the genitalia. Trypanosomes can be demonstrated in these lesions.

Consecutive passage of the attenuated strain through rats restores the virulence. This is accomplished more readily when the first passage is made with blood drawn shortly before death.

The successful production of an attenuated strain opens the possibility of immunizing animals against infection with *trypanosoma brucei*.

I wish to express my sincere gratitude to Professor F. G. Novy for his constant interest, advice, and many valuable suggestions during the pursuance of this work.

CLASSIFICATION OF PATHOGENIC STREPTOCOCCI BY FERMENTATION REACTIONS *

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The question of the unity or multiplicity of the streptococci, which arose soon after the first description of the organisms, is still unsettled. The recent work of Rosenow throws new light on the whole subject and indicates that if distinct species exist, rapid mutations from one to another may take place. The fact remains, however, that, as we meet them in nature, streptococci differ widely in their microscopic appearance, their manner of growth, their metabolic activities, and especially in their pathogenic properties. We meet with strains which grow on healthy or even abraded mucous membranes without any tendency to invade or to produce a reaction in the host. We meet with others which set up generalized infections of the most severe type, and with all gradations between these two extremes. Whether we regard these as distinct species or as variants of one species, it is of obvious importance to determine with which type we have to deal in any given case. Numerous tests have been devised to this end, but there seems to be no general agreement as to their value. Virulence for small animals does not seem to run parallel with that for human beings, and the classification according to hemolytic power, the method most generally used, is in some ways unsatisfactory as we shall show.

The application of the carbohydrate-splitting powers to classification, which proved of such value in the typhoid-colon-dysentery group of bacilli, has been attempted in the case of the streptococci in a desultory way by many observers, but very thoroughly by Gordon, Houston and Andrewes and Horder. The work of these English investigators has received scant attention, due perhaps to the complexity of Gordon's results. We were attracted to the subject by the reports of Winslow and Palmer¹ and others in this country who attacked this problem by making quantitative determinations of the acid produced in various sugars. They obtained results which differed somewhat from those of the English observers, and attributed their differences to the greater accuracy of their own methods.

The first extensive work on the fermentative reactions of streptococci was that of Gordon.² He first selected 10 strains of streptococci from various sources

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1. *Jour. Infect. Dis.*, 1909, 10, p. 288.

2. *Rep. Med. Off. Loc. Gov. Bd.*, 1903, 33, p. 388.

and tested the actions of these on 14 carbohydrates, 13 glucosides and 6 polyatomic alcohols. To many of these tests all strains reacted identically, and he selected 7 substances as of the greatest differential value, namely, lactose, saccharose, raffinose, inulin, salicin, coniferin and mannite. He also studied coagulation of milk, and the reduction of neutral red broth in anaerobic culture. These 9 reactions, since known as "Gordon tests," he then applied to 300 strains of streptococci isolated from 22 samples of saliva and found 48 groups in his 300 strains, which he indicated by letter and number (2a, 2b, etc.). There were, however, only 10 types of which 10 or more specimens were found. He also examined a few pathogenic strains. The characteristics of each strain were constant on repeated tests, except that a single animal passage in two instances altered the reaction (once to salicin and once to neutral red). Otherwise they remained constant.

Shortly afterward, Houston³ applied all the Gordon tests except that of coniferin to 300 strains of streptococci isolated from 20 human stools. He described 40 types differentiated by these reactions, but was able to bring about 60 per cent of the strains under 10 heads. The following year the same investigator⁴ reported tests made on 100 strains isolated from 10 specimens of cow dung. These showed, in general, some differences from the human type. For example: many of these fermented raffinose, whereas raffinose was attacked by very few of his human strains.

TABLE 1.
CLASSIFICATION OF STREPTOCOCCI BY ANDREWES AND HORDER

		Milk Clot	Neutral Red	Saccharose	Lactose	Raffinose	Inulin	Salicin	Coniferin	Mannite	Growth on Gelatin 20° C.	Morphology	Pathogenicity to Mice
A	Str. equinus	—	—	++	—	—	—	++	++	—	—	medius	—
B	Str. mitis	—	++	++	+	—	—	++	++	—	+	brevis	—
C	Str. pyogenes	—	++	++	+	—	—	++	++	—	++	longus	+
D	Str. salivarius	++	++	++	+	++	—	++	++	—	++	brevis	+
E	Str. anginosus	++	++	++	+	++	—	++	++	—	++	longus	+
F	Str. fecalis	++	++	++	+	+	—	+	+	+	+	brevis	+
G	Pneumococcus	++	—	+	+	+	++	—	—	—	—	brevis	+

Neither Gordon nor Houston suggested any satisfactory grouping of their numerous types, and the first attempt to clear the situation was made by Andrewes and Horder.⁵ They studied 288 strains from human lesions. Unlike Gordon and Houston's organisms, each strain was obtained from a distinct source (except in a few instances where more than one fermentative type was isolated from a sore throat or similar lesions, and so reduplication was avoided. The type of infection, caused by the organisms, gave them a clue to the significance of the metabolic tests. By systematic tabulation of their results and of those of previous workers, they were able to establish 7 types about which they grouped all the forms encountered. In addition to the Gordon tests they claimed differential value for the type of chain formation (longus, brevis and medius), the ability to grow on gelatin at 20 C., and the pathogenicity to mice. Their results are summarized in Table 1.

3. *Ibid.*, p. 472.

4. *Ibid.*, 1904, 34, p. 358.

5. *Lancet*, 1906, 2, p. 708, 775 and 852.

As these types will be referred to frequently, we may be permitted to describe them briefly. The authors insist that no sharp boundaries can be drawn between the groups, and that any tabulation of results must be tentative. As we will see later their grouping of aberrant types under the various heads may justly be questioned.

A. The streptococcus equinus, a saprophyte growing in the intestine of herbivora, consequently found in city air and dust; found occasionally in human feces and saliva; never pathogenic; characterized by inability to ferment lactose or to acidify milk. Most forms ferment saccharose and the glucosides, and a few ferment raffinose or reduce neutral red.

B. The streptococcus mitis, a short-chained form; almost never pathogenic; never found in suppurative processes; found frequently in saliva and human feces; grows well at 20 C.; does not coagulate milk; frequently reduces neutral red; ferments lactose and saccharose, frequently salicin and coniferin, and occasionally raffinose or inulin.

C. The streptococcus pyogenes, usually found in suppurative lesions and in septicemias. It is the most important pathogenic type and "in its fully developed form seems not to occur as a human saprophyte, though some of its variants have been met with in the saliva and feces. . . . It is a long-chained form usually growing in woolly masses at the bottom of a clear broth, occasionally the broth is more turbid and the chains only of medium length." It grows well at 20 C.; is actively hemolytic; does not clot milk or reduce neutral red; is pathogenic to mice and rabbits; ferments saccharose, lactose, and usually salicin. Any one of these reactions may be "suppressed," or other reactions may be added, and some variants are found which ferment coniferin or mannite, or liquefy gelatin, and a few variants ferment raffinose and inulin.

Andrewes and Horder regard this as a parasitic form which has evolved from Type B. It is not to be differentiated from the streptococcus mitis culturally, but only by the fact that it forms long chains, and is usually associated with suppurative processes.

D. The streptococcus salivarius, a short-chain form which clots milk and usually clouds broth, is the most common type found in the mouth; but also found often in human feces. It usually reduces neutral red; often fails to grow at 20 C.; ferments saccharose and lactose, usually raffinose, sometimes salicin or coniferin, and rarely inulin; is usually non-pathogenic, but otherwise closely related to the pneumococcus.

E. The streptococcus anginosus, found most frequently in the angina of scarlatina; seems to be a pathogenic form of the "salivarius"; its fermentative reactions are the same as in Type D, from which it differs in forming long chains, in failing to cloud broth, and in being actively hemolytic. In these respects it resembles Type C.

F. The streptococcus fecalis, found most often in the feces; practically never in the mouth, but is sometimes associated with inflammations, especially with cystitis; usually forms short chains and clouds broth, in which it produces H_2S ; ferments mannite always, saccharose, lactose, salicin and coniferin usually, and occasionally raffinose or inulin; it usually clots milk and reduces neutral red, and occasionally liquefies gelatin; it is not hemolytic.

G. Pneumococcus. Type characterized by the formation of a capsule under favorable conditions; the fermentative reactions are very variable, but it usually ferments saccharose, lactose and raffinose, frequently induces coagulation in milk, and ferments inulin; it rarely ferments salicin, coniferin or mannite, or reduces neutral red.

The analysis of the pathologic conditions, in which these various types are met, is of great interest, but for details one must refer to the original article.

Later, Gordon reviewed the work of Andrewes and Horder and accepted their classification;⁶ he also reported the results of the examination of 155 strains isolated from the throat in 50 cases of scarlatina.⁷ Organisms of the pyogenes type predominated, and were found in 38 of the 50 cases examined.

The classification given by Andrewes and Horder has much to recommend it, but the numerous \pm symbols in their table indicate that the classification lacks rigidity, and that a given strain is placed under one or under another head according to individual judgment.

Their results have not attained general acceptance. Walker⁸ found the fermentation reactions inconstant. According to his experiments, tests made on one and the same strain at intervals of weeks or months may give very different reactions on the Gordon media. By growing a strain in broth containing a sugar which it did not ferment for a number of generations, he was able in many instances to confer on it the ability to ferment this particular sugar. He was able to do this with a considerable number of strains, but not with all. On the other hand, after growing for several generations on milk, several strains lost their ability to coagulate milk. These results are at variance with those of other workers.

Buerger⁹ studied the fermentation of 34 strains, mostly pathogenic, but was unable to confirm Andrewes and Horder's results.

An attempt to classify streptococci by fermentative tests, quite independent of the work of the English investigators, was made by Salomon.¹⁰ This investigator examined the reaction of 78 organisms, streptococci, pneumococci and specimens of the streptococcus mucosus. He used 10 per cent. solutions of the test substances colored with litmus. Many of the reactions were of no differential value, but he found that glycerin, mannite, raffinose, arabinose and soluble starch were fermented by some strains and not by others. On the basis of these 5 reactions, he divided the pathogenic types into 4 groups:

"A. *Streptococcus pyogenes*.

I. *Streptococcus pyogenes* fermenting starch only.

II. Blood culture strains fermenting glycerin and mannite.

B. *Streptococcus mucosus*.

I. Fermenting glycerin, mannite and arabinose.

II. Not fermenting."

He also studied the fermentation of a number of strains in these 5 substances quantitatively by titration with phenolphthalein, but obtained no results of differential value.

Winslow and Palmer¹¹ based their work on the assumption that the change in color of litmus broth is not an accurate indication of the fermentative properties of bacteria. Streptococci form a slight amount of acid in most media, and the amount formed in those containing a fermentable sugar varies greatly with different types of these organisms. Their method was to plant a large series of strains in broth containing a certain carbohydrate. The amount of acid formed

6. *Jour. Path. and Bac.*, 1911, 15, p. 323.

7. *Rep. Med. Off. Loc. Gov. Bd.*, 1910, 40, p. 302.

8. *Proc. Roy. Med. and Chir. Soc., London*, 1910-11, 83 B, 541.

9. *Jour. Exper. Med.*, 1907, 9, p. 428.

10. *Centralbl. f. Bakteriol.*, 1908, 47, p. 1.

11. *Jour. Infect. Dis.*, 1910, 7, p. 1.

in 3 days was determined by titration, using phenolphthalein as an indicator—the acidity of uninoculated broth being subtracted from the result. The number of strains causing a given degree of acidity were then arranged in a frequency table and a curve plotted showing how many strains produced from 0 to 0.5 per cent. normal acidity; how many produced 0.5 to 1.0 per cent.; how many produced 1.0 per cent. to 1.5 per cent, etc. These curves as a rule showed two modes, one about zero or below, and the other above 1.0. Between these the curves dropped to a low point about 0.5 per cent., and this was taken as the dividing line, i. e., strains which produced 0.5 acidity or more were considered as fermenting; those which produced less, as non-fermenting. They used 3 of the Gordon media; lactose, raffinose and mannite broth, and also dextrose broth, and tested 116 strains of streptococci from human feces, 100 from horse dung, and 100 from cow dung. They grouped all of these, except a few aberrant types, in 9 classes. So far as could be determined from these tests, most of the strains correspond to some of Andrewes and Horder's types. Winslow and Palmer, however, subdivided these groups by the dextrose test (Table 2).

TABLE 2.
CLASSIFICATION OF FECAL STREPTOCOCCI (WINSLOW & PALMER.)

Carbohydrate Fermented	Name of Type (Andrewes and Horder)	Streptococci Found in		
		Man, Percent- age	Horse, Percent- age	Cow Percent- age
None				
Dextrose alone	{ Str. equinus	9 23	15 73	18 27
Dextrose and lactose	Str. mitis	31	5	21
Lactose and raffinose				
Dextrose, lactose and raffinose	{ Str. salivarius	0 5	0 0	12 9
Dextrose, lactose and mannit	Str. fecalis	23	0	2

The strains of the "equinus" type from man differed from those from the cow and horse in forming nearly twice as much acid in dextrose, and, in general, streptococci of human origin formed more acid in dextrose than other strains. The frequency curves for the human strains showed the mode at about 3.8 per cent., as against 1.8 per cent. for the equine, and 2.3 per cent. for the bovine strains.

The significance of these results is considerably lessened by the fact that like Gordon and Houston, Winslow and Palmer tested many "strains" from one source. Their 116, 100 and 88 strains were isolated from 15, 12 and 22 samples of human, equine, and bovine excreta, respectively. As many as 23 colonies were fished from a single specimen of feces and each was regarded as a separate strain, although many of them fermented in a practically identical manner. For example, the 23 per cent. of the streptococcus equinus from human feces was found in 5 of the specimens examined, and the 73 per cent. from the equine was found in 10 specimens.

Stowell and Hilliard¹² studied 70 strains from inflamed throats, from normal throats, and from milk. They titrated the acid formed in dextrose, lactose, maltose, raffinose, saccharose, and mannite, both at 37 C. and at 20 C. The results

12. *Amer. Jour. Dis. Child.*, 1912, 3, p. 287.

led to no definite classification. They met with surprisingly few raffinose fermenters, considering the source of their organisms, and with none which fermented mannite.

Broadhurst¹³ studied quantitatively the fermentations of 100 strains, isolated from 100 samples of cow's milk, on 6 carbohydrates—lactose, saccharose, salicin, raffinose, mannite, and inulin. She obtained much higher readings than Winslow and Palmer and found the "intermodal point" separating fermenters and non-fermenters to be about 1.5 per cent. She constructed frequency polygons from her own figures and from those published by Winslow and Palmer. Studied in this way, the milk streptococci resembled the human strains of Winslow and Palmer more than the bovine or equine. Qualitatively, the results were difficult to summarize. The strains fell into 19 groups, the largest containing 15 members. Only a few corresponded to the Andrewes and Horder types; but the majority may be described as atypical members of the following classes: the streptococcus fecalis, 27; the streptococcus anginosus, 8; the streptococcus pyogenes, 38; the streptococcus equinus, 23.

Following this, Winslow¹⁴ summarized all the work with quantitative titrations published. He concluded: "All these more or less preliminary results suggest that the biometric study of fermentative powers may not only throw light on the systematic relationships of the cocci, but may yield results of practical sanitary importance."

Stowell, Hilliard and Schlesinger,¹⁵ in a second paper, reported further studies of streptococci from milk and human throats. They arrived at no definite classification, but concluded that milk strains were characterized by high acid production, by ability to grow at room temperature, and by inability to ferment complex substances; that throat strains, on the other hand, usually fermented some more complex substances than the disaccharids at 37 C., but failed to do so at 20 C.

A recent paper by Fuller and Armstrong¹⁶ recorded a study of fecal streptococci which practically confirmed the results of Winslow and Palmer. They tested 349 strains on 7 media, using saccharin, salicin and inulin in addition to the media employed by Winslow and Palmer, but found the additional tests of little value in grouping the various strains.

In the second paper, Broadhurst¹⁷ compared the amount of acid produced in meat extract broth with that produced in meat infusion broth. She tested a number of strains against various carbohydrates, and found with few exceptions a much larger amount of acid in the infusion media than in the extract, when the sugar was fermented. One would infer that the infusion media giving better growth accentuate fermentative differences and are more favorable for this purpose.

We may summarize the facts gleaned from the literature as follows: The classification of streptococci according to fermentation reactions alone has led to the establishment of a bewildering number of types. Andrews and Horder grouped all these types under six heads to which they gave specific names. They showed a definite relation between their grouping by cultural tests and the pathogenicity and source of the organisms.

13. *Jour. Infect. Dis.*, 1912, 10, p. 272.

14. *Ibid.*, p. 285.

15. *Ibid.*, 1913, 12, p. 144.

16. *Ibid.*, 13, p. 442.

17. *Ibid.*, p. 404.

This classification has been attacked on several grounds: (1) Walker asserted that fermentation reactions were not constant in any strain, but vary from time to time; (2) Buerger found that such a grouping does not correspond to the type of pathogenicity of the strain; (3) Winslow and others in this country maintained that the quantitative determination of the acid produced was essential to an accurate study of fermentation. However, after very extensive work on saprophytic types they have arrived at no very definite results; (4) Salomon proposed a quite different grouping of the streptococci based on fermentation tests not used by Andrewes and Horder.

PLAN OF WORK

Three important questions remain unanswered. Is the ability to ferment carbohydrates and allied substances a constant characteristic of strains of streptococci as it is of members of the colon-typhoid-dysentery group of bacilli, and consequently a sound basis for classification? Is the amount of acid produced in fermentation constant, and of value? Does any constant relationship exist between the fermentation reactions, qualitative or quantitative, and the pathogenicity or source of the organism?

As much of the qualitative and practically all of the quantitative work had been done on saprophytic strains which are of little importance in medical bacteriology, we determined to use strains known to be pathogenic to man. We collected streptococci from as many types of lesions as possible.¹⁸ They were tested as soon as possible after isolation, most of them within two weeks, but some had been on artificial media for longer periods. The Neufeld bile-test was made on all specimens and only those strains which were bile insoluble were included in our studies. They were grouped as follows according to source: (1) Definitely pathogenic. Streptococcus strains obtained from blood of infected patients, or from closed suppurative processes; (2) probably pathogenic. Strains obtained in mixed culture from inflammatory exudates, for example, from crypts of inflamed tonsils; (3) saprophytic. Streptococcus strains from normal throats, from milk and from human stools. The fermentation reactions of such saprophytic races have been thoroughly studied by others as indicated above; but as the methods followed have been different in each case

18. Most of these were from patients in St. Luke's Hospital, but some were obtained through the courtesy of Dr. W. P. St. Lawrence, Dr. H. L. Celler, Dr. A. R. Dochez and Dr. D. S. D. Jessup.

it seemed advisable that we should include a few members of this group to determine whether the differences in method had any marked effect on the results. The streptococci in the first group were found in pure culture; those in the last two groups were carefully plated before making the tests. Our work on these micro-organisms has included studies of morphology, hemolysis, milk coagulation, reduction of neutral red, fermentation of carbohydrates and allied substances.

Our conclusions are based on the observation of these tests in 105 strains of streptococci. We will take up these various tests in order, discussing the methods used and the results obtained. We have also made fermentation tests on a number of strains of pneumococcus and of pneumococcus (or streptococcus) mucosus for comparison with the streptococci. These are included in Table 5 under separate heads.

I. MORPHOLOGY

We used only strains which showed chains of six or more members in broth, but accepted some in which diplococcus forms predominated, though occasional chains were present. We included in the series two strains which showed capsules when first isolated but which otherwise resembled streptococci. Our records (Table 5) are based on smears of sediment of twenty-four-hour dextrose broth cultures. Those forming chains of less than ten members are classed as brevis (B), those of ten to fifteen members as medius (M) and those of over fifteen members as longus (L). Three strains which formed very long chains covering several fields are designated as longior (LL).

The early attempts at streptococcus differentiation were based on morphology. Andrewes and Horder used it as a critical test and in their work the streptococcus mitis was differentiated from the streptococcus pyogenes and streptococcus salivarius from streptococcus anginosus chiefly by the length of the chains formed. We found, as have most observers, that the type of chain formation and the size of the individual cocci depend to a large extent on the medium and may vary considerably in a few generations. Long chains were often found in the sediment of a broth culture when pairs were present in the supernatant fluid. Moreover in most smears both long and short chains are found, so that it is difficult to make the readings objective. We found that most of the virulent pyogenic strains formed long chains and that most of those found in mild infections of the mucous membranes formed short chains. There were many exceptions, however.

We met with one morphological type which was fairly distinct. It grew in long chains of several hundred members. The dextrose broth cultures were perfectly clear with a massive gelatinous sediment which was difficult to break up. This type occurs in the mouth and is only slightly pathogenic. We found three typical examples. The fermentation reactions were not the same, and consequently they could not be grouped together in our table. Moreover, we found several very long chain formers which approached this type in morphology and cultural appearance, and one strain which formed chains of about twelve when first isolated, but later grew in chains of fifty or more, and formed a heavy gelatinous sediment. Probably, then, these long chain forms are not a distinct variety but a manner of growth which more than one variety may assume. We do not think, therefore, that morphology can be used as a basis for classification of streptococci.

II. HEMOLYSIS

The observation of hemolysis on blood agar plates introduced by Schottmüller is the most widely used means of differentiating streptococci. We examined most of our strains by making surface streaks on human blood agar and observing them from one to three days. Some strains, notably the mannite fermenters, produced no lysis. Others obtained from septicemias produced a wide zone in less than twenty-four hours. Between these two extremes, however, were all gradations. Many of the pneumococci and strains of the "viridans" type from endocarditis produced a narrow zone in two to three days. The hemolysis depended also on the depth of the medium and the amount of blood it contained, for in an unevenly mixed blood plate it was sometimes observable in the thin, but not in the thicker portions. Moreover a few strains which were fished from colonies surrounded by a narrow cleared zone, and which came from throats, did not later show any hemolytic power. It is difficult, then, to draw a line sharply between hemolyzing and non-hemolyzing types. The property is present to some extent in most streptococci and while in general it is more pronounced in the more virulent type, the differences are essentially quantitative and therefore not altogether satisfactory as the basis of classification. We have attempted to indicate the strength of hemolysis by one, two or three plus marks in our table, but the estimations are necessarily inaccurate.

III. MILK COAGULATION

Milk coagulation was determined by observations on litmus milk after five days' incubation. We found that this property was not constant in all strains, for some acquired it while under observation. It was roughly parallel to high acid production in lactose broth though a few strains were found which formed only small amounts of acid but still coagulated milk. In general, the strains which coagulated milk were of low pathogenicity, and vice versa; yet there were many exceptions, that is, Strain 158, obtained from a rapidly fatal septicemia, coagulated milk in twenty-four hours. Andrewes and Horder consider this one of the most significant tests and their differentiation between the streptococcus pyogenes and streptococcus anginosus is based chiefly on this criterion. The fact that it is not constant to a given strain, however, makes it of little value.

We met with three strains which promptly coagulated the milk, and then peptonized it ("P," Table 5). They also liquefied gelatin, and were probably the type described by MacCallum and Hastings as the micrococcus zymogenes.¹⁹ The three strains did not show identical fermentation reactions.

IV. REDUCTION OF NEUTRAL RED

We made this test in 1 per cent. dextrose-neutral-red broth covered with liquid petrolatum. Only five strains reacted. We have not found a description of the technic used by others in making this test, but as our results differed so widely from theirs it seemed unlikely that our method was the same. The tests as done by us were of no assistance in classification.

V. FERMENTATION OF CARBOHYDRATES AND ALLIED SUBSTANCES

As this was the part of the work which chiefly interested us, we will describe our methods in some detail and then discuss our results: (1) as to the qualitative study of the presence or absence of fermentation in the various test substances; and (2) as to the quantitative study of the amount of acid produced.

METHODS

The media used in our first tests were made from veal infusion broth containing 1 per cent. peptone (Witte) and 0.5 per cent. sodium chlorid adjusted to about 0.3 per cent. acidity. To this was added 1 per cent. of the fermentable sub-

19. *Jour. Exper. Med.*, 1899, 4, 521.

stance to be tested. As many of our strains grew feebly in this medium, we later adopted 2 per cent. peptone broth which seemed more favorable. The tubes were inoculated at first with a loop-full of growth from a twenty-four-hour agar or ascitic-agar slant—later with a few drops of a twenty-four-hour dextrose broth culture from a capillary pipet. The latter method gave more constant results. The tubes were incubated for twenty-four hours and 5 c.c. of the contents titrated against N/20 normal NaOH, using the first pink of phenolphthalein as the end reaction. The residue was tested by the addition of a few drops of litmus solution. A smear from one or more tubes was examined to determine whether growth had taken place. All the tests were made in duplicate, and when there was any material discrepancy, they were repeated as many times as seemed necessary. A control tube of each lot of every carbohydrate was titrated and the percentage of acidity deducted from that of the inoculated tubes. A second correction was made by inoculating a tube of plain broth (as a rule from the same lot of meat infusion as the sugar broth) and deducting the amount of acid formed from the result. This procedure was adopted to avoid the use of colon free-sugar broth recommended by some investigators, partly because many streptococci grow feebly in colon broth, and partly to exclude possible acid formation from non-carbohydrate constituents. The twenty-four-hour period was chosen because very little acid is formed after this time, as shown in Table 3. Two low fermenting strains and two high fermenting strains were tested in a dextrose broth after incubation for varying periods, and the readings after thirteen hours were about the same as after five days.

TABLE 3
RAPIDITY OF ACID PRODUCTION BY FOUR STRAINS OF STREPTOCOCCI

	13 Hours	29 Hours	53 Hours	73 Hours	120 Hours
Strain 108.....	4.8	5.7	6.1	5.6	6.3
Strain 157.....	5.2	5.7	5.6	4.5	5.4
Strain 9.....	3.6	3.4	3.4	3.7	2.8
Strain 158.....	3.5	3.4	3.4	...	3.6

Only the results obtained from 2 per cent. peptone media were used in plotting frequency curves, as these ran from 0.3 per cent. to 1.2 per cent. higher than those obtained on 1 per cent. peptone. Where both of the duplicate tests were made on this medium, the average of the results was recorded. Where the first test was made on 1 per cent. peptone broth and the results on 2 per cent. agreed qualitatively, the latter figures were used in constructing Table 5. A number of strains tested only on 1 per cent. peptone and starred (*) are included in our table.

The fermentable substances used were the "Gordon test media," with the changes recommended by Stowell and Hilliard, namely; dextrose; lactose, saccharose; salicin, raffinose, mannite and inulin. Later, to compare our results with those of Salomon, glycerin, soluble starch and arabinose were used. The lactose, salicin, raffinose, glycerin and starch used were Merck's. The saccharose was also Merck's "reagent." The mannite was Kahlbaum's; the dextrose, Eimer & Amend's c. p.; the inulin, Eimer & Amend's "Kiliani." Part of the arabinose tests were done with sugar of Kahlbaum's preparation; the others with a preparation obtained from Eimer and Amend. For the litmus test, we used Merck's "reagent" grade in 1 per cent. solution.

QUALITATIVE RESULTS

All tubes in which 0.8 per cent. normal acidity or more was produced were considered positive, and all less than this, negative, for reasons which we will discuss in considering the quantitative results.

Are these results constant in any given strain? From our observations we are unable to assert definitely that they are constant, but what evidence we have obtained points in that direction. We have re-examined twenty-four of our strains at intervals of eight to thirty-four weeks and found the reactions qualitatively unchanged. In other instances we have found discrepancies which are difficult to explain. Not infrequently a reaction was found negative on a single test which later was consistently positive. This we attributed to feebleness or absence of growth in the unfermented tubes. In a few instances, fermentation was obtained on the first test but not on subsequent tests, even though frequently repeated. We can account for these latter cases only by assuming some error, such as a misplaced label or contamination of the tubes. Most of the inconsistencies were in the non-hemolyzing organisms which grew feebly on most of our media. One of the most puzzling examples were the results with Strain 126 on salicin which were as follows: May 31, 1.4; June 26, 0.3; June 27, 0.4; September 16, 1.1; September 16, 2.5; September 18, 2.8.

Strain 125, of the "viridans" type, failed to ferment lactose on the first two tests, and grew feebly. In ascitic fluid-lactose broth it gave a positive reaction and when two plain lactose broth tubes were subsequently inoculated with large amounts of a young culture, it gave positive fermentation in both. These were exceptional cases. The latter may be an instance of acquired fermentative property, but more probably of adaptation to the new environment—the streptococcus fermenting the broth as soon as it was able to grow actively on it. Obviously an organism will not ferment a broth in which it will not grow, and apparently it may also grow feebly in a broth too unfavorable to permit fermentation. The best illustration of the effect of the unsuitability of the medium on fermentation was in the pneumococci. Some strains which repeatedly failed to ferment inulin broth in which they appeared to grow gave typical fermentation in Hiss' inulin serum water. Broadhurst has shown also that more acid is produced in infusion-broth than in extract. The fact that our strains fell into a small number of groups more completely than those of other observers,

we attribute to the use of richer media. Reactions might well be missed in the extract broth or colon sugar-free broth which many have used, as they are poor media for streptococci. We have not used the method of Walker, that of growing a strain for a number of generations on a non-fermentable sugar to produce a positive reaction. We believe that further work is necessary to determine the permanency of the fermentative characteristics. However, in the great majority of instances the results of repeated tests have agreed, and in no instance (except possibly the one cited, Strain 125) could we determine that a consistently negative reaction became consistently positive, or the reverse. Even if it is proven that it is possible to change the reactions of streptococci by artificial methods, this does not necessarily destroy the practical value of the tests. Twort²⁰ has claimed that by methods similar to those of Walker, mentioned above, he could alter typhoid bacilli so that they would ferment lactose, but, though this be true, it is well established that failure to ferment lactose is characteristic of typhoid bacilli as found in nature.

After we had concluded that these reactions were sufficiently constant to be of value, our next consideration was to determine whether there was any relation between the fermentative powers of a given streptococcus and its pathogenicity or source. We determined to disregard other tests and group them strictly according to their fermentation reactions and then see whether we could correlate such a grouping with our information in regard to their origin. Some of the tests we discarded because, for instance, dextrose was fermented by every strain we tested. Only three showed a positive reaction in glycerin. (Two of these also fermented the hexahydric alcohol mannite.) None of those tested on arabinose fermented it. Starch was fermented by fourteen strains. Seven of these agreed in their other fermentative properties and in their pathogenic properties with the pyogenic type described below, while others did not. Consequently we also discarded this test in defining our groups. On the basis of the remaining tests nearly all the strains examined fell into six groups. On examining the sources from which the cultures in these groups were obtained, we were able to characterize one fermentative group as pathogenic and the others as essentially saprophytic. Members of four of the saprophytic groups were found chiefly in the mouth and

20. Cited from Penfold, *Brit. Med. Jour.*, 1910, 2, 1672.

throat, and those in the remaining group in the intestines. The classification arrived at, was as follows:

A. Pathogenic type:

Group 1, fermenting lactose, saccharose and salicin.

B. Saprophytic types:

a. Salivary types:

Group 2, fermenting lactose, saccharose and raffinose.

Group 3, fermenting lactose, saccharose, salicin and raffinose.

Group 4, fermenting lactose, saccharose, raffinose and inulin.

Group 5, fermenting lactose and saccharose.

b. Fecal type:

Group 6, fermenting lactose, saccharose, salicin and mannite.

Thirteen of the strains examined gave other combinations of fermentation reactions than those given in the above table. Seven of these strains fermented inulin, and for this reason we have classified them under Group 4. Of the remainder, three fermented mannite and were classified under Group 6, and three strains were left unclassified.

Group 1. An examination of Table 5, which gives our series of streptococci grouped in this way, shows that all the highly pathogenic forms, with a few exceptions which we will consider later, fell into Group 1, which we have called the pyogenic group. It includes nine strains from acute septicemia, eleven strains from abscesses and eleven strains from other severe suppurative infections. In this respect our results agree with those of Andrewes and Horder for all the streptococci from abscesses and suppurating wounds which they examined gave these same fermentative reactions. This same type was found in the most severe cases of tonsillitis which we examined, and was recovered from the tonsillar crypts of one of our patients three months after recovery. With this exception, it was not found in normal throats. In a few instances, streptococci of the pyogenic type were found in milder infections of the nose, mouth, throat and bronchi; also once in milk, and once in the air. Most of these pyogenic streptococci grow in long chains, hemolyze blood agar, and do not coagulate milk, but these characteristics are not common to all members of the group.

The remaining types seemed essentially saprophytic. Members of Groups 2, 3 and 4 were found in normal throats. They were also frequently associated with mild infections such as chronic tonsillitis and pyorrhea alveolaris. One of these types (2) occurred in a mild otitis media in a poorly nourished child. The only severe type of infection, in which streptococci belonging to these groups were concerned, was endocarditis. In this disease they were found in many instances. These groups include both long and short chain types. Most of the organisms cause little or no hemolysis on blood agar and the majority coagulate milk.

Group 5 includes streptococci occurring as intestinal saprophytes. These races were found in a few severe processes (cystitis, prostatic abscesses, and puerperal sepsis) in which infection from feces seemed probable. They were found also in otitis media (twice) and in infectious endocarditis (twice). Other observers have agreed in considering mannite-fermenting streptococci characteristic inhabitants of the human intestine, and Andrewes and Horder met with them in the same types of infections as did we. These streptococci usually grow in short chains and are not hemolytic. About half of the strains coagulate milk.

Many of the streptococci which we have placed in Groups 2, 3 and 4 answer to the description of the streptococcus salivarius given by Andrewes and Horder and those in Group 5 correspond to the streptococcus fecalis of these investigators. We prefer the grouping given above because it follows rigidly the fermentation tests which are more constant than the characteristics on which they base the differentiation of their types, (viz., morphology and milk coagulation). We were unable to confirm in any respect the findings of Salomon.

So far as we could determine by our observations, a useful differentiation between several distinct types of streptococci can be made by determining qualitatively their ability to ferment lactose, salicin, raffinose, mannite and inulin. Further study is required to determine whether or not tests on saccharose and soluble starch are of value.

QUANTITATIVE RESULTS

Winslow and Palmer claimed for their titration method, (1) that it gave a more accurate verdict than the litmus test in deciding whether or not the test substance in question is fermented; and (2) that a

comparison of the amount of acid formed by various streptococci is a sound basis for classification.

To test the correctness of their first conclusion we have made frequency tables and curves for each fermentable substance used. In each case in which an "intermodal point" between fermenting and non-fermenting groups could be determined, it fell in the class "0.6 per cent. to 1.0 per cent." Consequently we have made our division at 0.8 per cent. and considered the streptococci which produced 0.8 per cent. normal acidity or more as fermenting; others as non-fermenting. On comparing these results with those of the litmus test we found that they were practically identical. The change in the color of litmus from blue to pink was complete in the broth we used at a point 1.2 per cent. to 1.4 per cent. acid to phenolphthalein. In recording our results about 0.7 per cent. was usually subtracted from our readings to correct for the initial acidity of the medium and the acid produced in plain broth. Therefore, in a tube which was distinctly acid to litmus and no more, the corrected reading by titration would be about 0.7 per cent. In short, dropping litmus solution into the tube seemed quite as satisfactory a means for determining whether or not fermentation had taken place as titrating the contents with phenolphthalein.

In considering the value of our quantitative determinations as a basis for classifying the various races, we inquired (1) as to what determines the amount of acid which a streptococcus will produce in a given medium; (2) whether this is a constant property of each strain; and (3) whether this property could be correlated with the pathogenicity, the source, or the qualitative fermentation reactions of the organism.

In regard to the first question it seemed that the production of various degrees of acidity by different streptococci might depend either on a difference in the end products of the fermentation in each case, so that one organism produces more acid (more free H ions) from a decigram of sugar than another, or it might depend on a difference in sensitiveness to the acidity of the medium, that is, one organism might be inhibited in its growth or ferment activity by a lower acidity than another.

Stowell and his coworkers take the former view. They say: "The streptococci never carry a fermentation through to the simple elements, carbon dioxid and alcohol, and so we must make our measurements of the amount of fermentative activity that they exert by determining

the amount of acid they split off from the molecule. . . . It is this varying capacity of different bacterial strains to separate uniting bonds existing between atoms and groups of atoms and thus avail themselves of the energy liberated in the process, which we try to measure by acid titration." They present, however, no evidence whatever for the correctness of this rather fanciful hypothesis.

To test the correctness of their theory we planted two low fermenting and two high fermenting strains on broths containing 4 per cent., 2 per cent. and 1 per cent. of dextrose. If the degree of acid produced by each race were dependent only on "the amount of acid they split off from the molecule," it should be proportional to the amount of sugar available. As is shown in Table 4, the acidity was substantially the same regardless of the percentage of sugar.

TABLE 4.
FOUR STRAINS GROWN IN BROTHS OF VARIOUS ACIDITY AND CONTAINING VARIOUS AMOUNTS OF DEXTROSE

Percentage of Dextrose	0	4%	2%	1%	1%	1%	1%
Initial acidity	0.6	0.5	0.5	0.5	2.6	4.3	6.4
Acidity produced in 24 hours							
Strain 108.....	0.6	5.8	5.1	5.3	5.0	4.5	6.6
Strain 157.....	0.6	4.9	4.9	4.8	4.7	3.5	6.5
Strain 9	0.6	2.9	2.9	3.3	2.7	4.5	6.7
Strain 158.....	0.7	2.4	2.6	2.5	2.6	4.3	6.9

From these observations we concluded that the acidity of the broth was limited by some factor which inhibited fermentation—probably the acid itself. To determine this we added varying amounts of sterile normal lactic acid to 1 per cent. dextrose tubes and then inoculated them with the same four strains (Table 4). The final reaction in all the tubes inoculated with each strain was the same (within the limits of error of our method), which shows that fermentation by a given streptococcus ceases when a certain acidity is reached, irrespective of how much acid must be formed to produce this acidity. All we can determine, apparently, by the titration method is the sensitiveness of a particular streptococcus, or its enzyme to acid.

This property might still be of value if it were constant in a given strain. We found, however, that the acidity produced by the same strains in the same medium varied for reasons which we were unable to discover. The results from duplicate tests sometimes checked very closely, especially if made from the same seed tube at the same time; for example:

	Arabinose	Glycerin	Starch	Inulin	Mannit	Raffinose	Salicin	Saccharose	Lactose	Dextrose
Strain 130, Sept. 22.....	4.9	4.5	4.8	4.3	4.3	3.2	3.1	2.4	0.0	0.2
Strain 130, Sept. 22.....	4.7	4.5	4.6	4.6	4.3	3.2	3.1	2.4	0.1	0.0

This was not always the case, however, as is shown in Table 3. Strain 157 which was planted simultaneously on several tubes of 1 per cent. dextrose broth produced only 4.5 per cent. acid in the tube incubated for three days, while those incubated two days and five days titrated 5.6 per cent. and 5.4 per cent. respectively. If duplicate tests were made of the same strain at intervals of several days, rather large variations were apt to occur, for example:

	Dextrose	Lactose	Saccharose	Salicin	Raffinose	Mannit	Inulin	Starch	Glycerin	Arabinose
Strain 112, Aug. 26.....	3.4	4.5	4.7	4.1	0.7	0.7	4.3	0.7	0.7	0.4
Strain 112, Aug. 28.....	3.1	3.8	3.6	2.4	0.6	0.4	3.0	0.4	0.5	0.7

Notwithstanding the fact that the amount of acid produced by a given strain may vary considerably, we grouped the streptococci in our series on the basis of our quantitative studies. We found that in general highly pathogenic strains produced less acid than saprophytic strains, and that the streptococci causing endocarditis produced more acid than the other definitely pathogenic types. There were, however, numerous exceptions to these generalizations, and we were unable to arrive at any satisfactory classification by this method. A comparison of our frequency tables with those for saprophytic streptococci published by other workers also led to no results. In short, we derived no useful information from our titrations that could not have been obtained by the qualitative litmus test.

CONCLUSIONS

Analysis of our experiments seems to justify the following conclusions:

1. The streptococci usually concerned in severe infections in man may be differentiated from the common saprophytic types by fermentation tests.

Serial No.	Source	Pathogenicity	Dextrose	Lactose	Saccharose	Saltin	Raffinose	Mannite	Inulin	Starch	Glycerin	Arabinose	Morphology	Milk	Neutral Red	Hemolysis
Group 1, Pyogenic Type																
4	Blood, sepsis	+	22	25	24	28	0.4	-1	0.2	0.5	0.3	...	L	0	0	+
10	Blood, sepsis	+	29	25	12	22	-1	-1	0.1	0.5	0.2	...	B	0	0	+
22	Blood, sepsis	+	21	26	26	22	0.5	0.1	0.4	0.3	0.1	0.3	B	0	0	+
61	Blood, sepsis	+	26	25	30	31	0.1	0.0	0.3	0.6	0.1	...	L	0	0	+
143	Blood, sepsis	+	10	11	15	13	0.2	0.2	0.0	0.0	0.2	...	L	0	0	+
145	Blood, sepsis	+	42	37	36	32	0.1	-2	0.4	0.0	0.2	-1	M	0	0	+
103	Blood, sepsis	+	26	24	27	17	0.2	0.0	0.1	0.2	0.2	-6	M	0	0	0
158	Blood, sepsis	+	12	12	15	10	0.2	0.0	0.1	0.4	-1	0.0	M	0	0	+
20	Blood, sepsis	+	22	22	24	16	0.1	0.3	0.1	2.2	0.3	0.0	M	0	0	+
26	Blood, pyemia	+	11	12	14	13	0.2	0.3	0.1	0.5	0.3	...	B	0	0	+
59	Pus, abscess	+	25	18	16	12	0.6	0.3	-1	0.0	0.3	...	B	0	0	+
72	Pus, abscess	+	28	24	24	22	0.4	0.2	0.0	0.9	0.2	...	L	0	0	+
90	Pus, abscess	+	22	20	26	15	0.4	0.2	0.0	0.4	0.4	...	L	0	0	+
102	Pus, abscess	+	17	19	26	13	-2	0.1	0.2	0.1	0.1	-3	L	0	0	+
119	Pus, abscess	+	13	12	16	16	0.3	-1	0.2	0.0	0.1	-4	L	0	0	+
23	Pus, abscess	+	18	21	26	11	0.3	0.0	0.2	0.0	-2	0.0	B	0	0	+
32	Pus, abscess	+	13	33	12	12	0.2	-1	-1	0.5	0.6	0.5	L	0	0	+
70	Pus, abscess	+	11	19	13	13	0.3	0.5	0.3	0.9	0.3	0.1	M	0	0	+
10	Pus, liver abscess	+	13	16	12	17	0.3	-1	0.3	0.1	0.3	-2	L	0	0	+
16	Pus, empyema	+	17	17	24	27	-4	-4	-3	0.2	-1	0.0	B	0	0	+
36	Pus, empyema	+	23	26	27	27	0.0	0.1	-3	0.2	-1	-2	L	0	0	+
33	Pus, pericarditis	+	21	26	27	40	0.3	-5	0.3	-2	0.0	-2	L	0	0	0
83	Pus, pericarditis	+	39	44	23	40	0.3	0.7	0.0	0.3	0.1	0.0	B	0	0	0
82	Pus, meningitis	+	2.2	29	24	24	0.3	0.2	0.5	0.3	0.2	-2	L	0	0	+
85	Pus, meningitis	+	19	24	25	24	0.4	0.1	0.3	0.5	0.2	-2	L	0	0	+
40	Pus, meningitis	+	19	21	20	14	0.2	0.2	0.2	2.3	0.1	-2	M	0	0	+
18	Pus, otitis media	+	19	20	20	14	0.3	0.1	-4	0.2	0.1	...	B	0	0	+
19	Pus, otitis media	+	20	24	24	25	-1	0.2	0.4	0.2	0.3	...	B	0	0	+
28	Pus, folliculitis	+	20	25	0.8	1.3	0.3	0.1	-2	-2	-1	...	B	0	0	+
78	Gangrene of leg	+	14	12	13	1.9	0.2	0.2	0.1	B	0	0	+
13	Pus, wound infection	+	17	39	16	1.5	0.3	0.2	0.1	0.6	0.1	0.5	L	0	0	+
124	Blood, endocarditis	+	37	39	41	28	0.1	-1	-2	-0	-1	-1	...	0	0	0
126	Blood, endocarditis	+	3.2	32	29	27	0.2	0.0	-1	0.1	0.1	0.1	B	0	0	+
46	Blood, endocarditis	+	1.6	12	18	12	0.4	0.1	0.5	0.3	0.0	...	L	0	0	+
49	Tonsil, acute tonsillitis	+	2.6	25	32	27	0.1	-2	0.2	0.2	0.2	-1	L	0	0	+
111	Tonsil, acute tonsillitis	+	1.8	25	20	24	0.1	0.0	0.3	0.2	0.2	0.5	L	0	0	+
8	Tonsil, acute tonsillitis	+	1.0	1.0	0.9	1.1	-1	-3	-2	0.6	0.6	...	L	0	0	+
104	Throat, acute pharyngitis	+	1.8	1.4	2.2	2.0	0.4	0.0	0.1	0.0	0.0	-2	B	P	0	+
31	Sputum, acute pharyngitis	+	4.1	40	40	33	0.6	0.5	0.3	0.1	0.5	-1	M	+	0	+
17	Throat, diphtheria	+	1.9	27	27	3.1	0.6	0.4	0.7	0.4	0.6	...	L	+	0	+
42	Sputum, pertussis	+	1.9	28	21	2.5	0.3	0.1	-2	-2	0.0	...	L	+	0	+
118	Sputum, bronchiectasis	+	1.9	1.9	2.1	1.0	0.2	-2	0.1	0.0	-1	0.6	LL	0	0	+
50	Sputum, bronchitis	+	1.2	1.2	1.6	1.2	0.5	0.7	0.2	0.0	LL	0	0	+
12	Tonsil, chronic tonsillitis	+	2.3	2.5	2.6	2.6	0.2	0.0	-1	0.2	0.2	0.5	B	0	0	+
23	Tonsil, chronic tonsillitis	+	2.4	3.4	2.8	2.6	0.3	0.4	-1	0.1	0.2	...	L	+	0	+
66	Tonsil, chronic tonsillitis	+	1.7	1.9	2.2	0.9	0.0	0.0	-2	2.4	0.0	...	B	0	0	0
114	Nose, sinusitis	+	1.8	2.2	2.8	2.2	0.2	0.3	0.0	0.4	0.3	0.2	L	+	0	+
27	Gum, pyorrhea	+	2.2	2.6	2.8	2.7	0.5	0.2	0.4	2.9	0.3	0.1	L	+	0	+
15	Air	+	2.6	0.9	1.2	1.8	0.1	-1	0.3	0.4	0.3	0.3	B	+	0	0
109	Milk	+	6.1	4.7	5.2	3.9	0.4	0.1	0.1	0.3	0.1	-2	L	+	0	+
155	Tonsil, normal	+	1.9	2.5	1.3	2.4	0.1	-4	0.2	0.3	0.4	0.1	L	+	0	+

TABLE 5.—(Continued)

Serial No.	Source	Pathogenicity	Dextrose	Lactose	Saccharose	Salicin	Raffinose	Mannite	Inulin	Starch	Glycerin	Arabinose	Morphology	Milk	Neutral Red	Hemolysis
(GROUPS 2 AND 3, SALIVARY TYPES FERMENTING RAFFINOSE)																
53	Pus, otitis media.	P	3.1	2.5	1.7	-2	2.1	-2	0.4	-1	-1	...	L	+	0	+
123	Blood, endocarditis	P	2.5	2.6	2.8	0.7	3.9	.2	-1	0.0	0.0	0.0	L	+	0	+
137	Blood, endocarditis	P	2.1	2.6	3.2	-1	2.7	-1	0.1	0.0	0.0	0.0	B	+	0	+
57	Sputum, asthma	?	2.0	2.3	1.3	-2	1.9	-5	0.4	0.2	0.3	...	B	+	0	+
81	Gum, pyorrhea	?	2.8	2.6	3.0	-2	2.6	0.0	0.1	0.0	0.0	-1	B	+	0	+
106	Tonsil, chronic tonsillitis	?	3.6	3.0	3.2	0.3	2.7	-2	0.1	-2	0.1	-3	B	+	0	+
26	Tonsil, chronic tonsillitis	?	4.3	4.6	4.9	5.3	4.9	0.1	0.1	0.3	0.1	...	B	+	+	+
29	Gum, pyorrhea	?	4.1	4.0	4.2	3.9	3.8	0.0	0.1	0.1	0.1	...	B	+	+	+
51	Sputum, bronchitis	?	1.5	1.9	2.6	1.7	2.4	0.2	0.0	2.3	0.2	...	L	0	+	+
67	Tonsil, chronic tonsillitis	?	2.1	2.4	2.6	1.9	2.1	0.1	0.2	2.7	0.3	0.0	M	+	0	0
137	Tonsil, normal	S	4.5	3.7	3.8	0.6	4.7	0.1	0.2	0.1	0.1	-2	M	+	0	0
138	Tonsil, normal	S	4.8	4.5	5.2	5.0	4.0	0.0	0.0	0.2	-1	-2	M	+	0	0
140	Tonsil, normal	S	4.8	3.6	3.2	3.3	2.9	-1	0.0	0.1	-2	-2	M	+	0	0
142	Tonsil, normal	S	5.4	2.9	5.1	3.7	3.0	0.0	0.4	0.2	0.0	0.0	B	+	0	+
(GROUP 4, SALIVARY TYPE FERMENTING INULIN)																
144	Blood, endocarditis	P	3.3	2.0	3.4	3.4	0.1	-3	3.3	0.0	-1	0.0	B	+	0	0
130	Blood, endocarditis	P	4.5	4.1	4.3	4.1	3.9	2.8	2.1	2.0	-3	-2	M	+	0	0
127	Blood, endocarditis	P	4.2	4.2	3.7	3.7	3.0	1.6	2.5	0.0	0.0	0.3	M	+	0	0
68	Sputum, bronchitis	?	1.6	1.5	2.2	-1	1.3	-1	0.9	0.0	0.2	...	L	+	+	+
101	Sputum, bronchitis	?	3.0	3.1	3.6	3.5	2.1	-2	1.9	.1	0.0	-6	L	+	+	+
155	Gum, pyorrhea	?	5.4	5.8	5.6	5.0	0.5	0.1	2.3	0.3	0.1	0.7	B	+	0	0
96	Sputum, pneumonia	?	2.5	2.7	2.9	0.0	2.6	0.1	0.8	0.4	0.3	-2	B	+	+	+
112	Tonsil, normal	S	2.9	3.8	3.8	2.9	0.3	0.2	3.3	0.2	0.2	0.2	B	+	0	+
113	Tonsil, normal	S	2.9	2.9	1.9	1.5	3.0	0.0	2.1	0.5	0.2	0.0	L	0	0	+
143	Tonsil, normal	S	3.1	2.6	2.6	-1	2.8	0.2	2.3	0.6	0.0	-1	M	+	+	+
141	Tonsil, normal	S	3.6	3.0	3.3	0.1	3.1	-1	1.9	0.2	0.0	-2	L	+	0	0
(GROUP 5, SALIVARY TYPE NOT FERMENTING SALICIN, RAFFINOSE, MANNITE OR INULIN)																
13	Blood, sepsis meningitis	P	2.5	2.7	2.0	0.2	0.4	0.3	0.0	0.2	0.3	...	L	0	0	+
122	Blood, endocarditis	P	2.7	1.8	2.4	0.0	0.1	-2	0.0	-1	0.0	0.2	L	+	0	+
125	Blood, endocarditis	P	2.8	2.9	3.0	0.1	0.0	-5	-4	-3	-3	0.1	L	+	0	0
129	Blood, endocarditis	P	3.1	2.2	2.7	-1	0.5	0.0	-2	0.3	0.1	0.4	B	+	0	0
131	Blood, endocarditis	P	3.1	3.0	3.9	-2	0.4	0.1	-1	0.0	-2	0.0	L	+	0	0
151	Chancroid	?	2.2	2.5	2.2	-2	0.2	0.0	0.0	0.7	0.1	0.3	B	+	0	0
11	Gum, pyorrhea	?	2.2	3.6	1.9	-2	-3	-3	-2	-3	-3	...	B	+	0	+
25	Gum, pyorrhea	?	1.6	1.8	1.9	0.5	0.4	0.3	0.2	B	+	0	0
110	Tonsil, chronic tonsillitis	?	4.2	3.7	3.7	-4	-1	-4	0.0	0.0	-2	-6	B	+	0	0
60	Eye, conjunctivitis	?	2.0	1.7	1.7	-1	1.1	-1	0.0	0.0	0.0	-1	L	0	0	0
115	Sputum, normal	S	3.4	3.1	2.8	-2	0.1	-1	-2	0.0	-1	-1	B	+	0	+
43	Milk	S	2.0	1.9	2.4	0.1	0.3	0.3	0.3	2.1	0.3	...	L	+	0	+

2. The tests are best performed in infusion broth colored with litmus, containing 2 per cent. or more of peptone and 1 per cent. of the fermentable substance. The initial reaction should be between 0.0 and 0.5 per cent. normal acidity (phenolphthalein). Only a complete change of the litmus to pink should be considered positive. The useful test substances are lactose, salicin, raffinose, mannite and inulin.

3. On the basis of these reactions we can recognize one pathogenic and six saprophytic groups:

(a) Pyogenic group; characterized by fermentation of lactose and salicin.

(b) Four salivary groups; two characterized by fermentation of raffinose, one by fermentation of inulin and one by failure to ferment salicin, raffinose, mannite or inulin.

(c) Fecal group; characterized by fermentation of mannite.

(d) Equine group; characterized by failure to ferment lactose.

We mention this last group, with which we have had no experience, for the sake of completeness. Its existence seems well established by the work of other investigators. The streptococci in this class are saprophytes and are commonly found in horse dung.

4. Titrations of the amount of acid formed in fermentation are of no value in classification.

5. Tests for ability to hemolyze human blood are of value in that highly pathogenic strains are usually strongly hemolytic.

6. The type of chain formation, the milk coagulation test, and the neutral red reaction, are not reliable as bases for classification.

These conclusions, being based on observation of only 105 strains, are necessarily tentative. The validity of the fermentation tests depends chiefly on whether or not they remain constant in a given race. Work along the lines opened up by Rosenow may show that we can artificially alter the fermentative powers of streptococci as well as their other properties. It is our purpose to preserve some of our strains for a year or more on artificial media to determine whether their fermentative powers change. We believe, however, that the reactions that we have described are characteristic of certain types of streptococci as they are isolated from human disease.

ANAEROBIC CULTURES IN SCARLET FEVER *

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PLATE I

The association of streptococci with scarlet fever has been so constant a finding as to be suggestive of an etiological relationship. It seemed, however, desirable to make a search for organisms by newer cultural methods, those which have usually been used being poorly adapted to the culture of many bacteria. Hence blood-cultures were made by different anaerobic methods in a series of 24 cases. Only 4 of these cultures were negative. As soon as it was found that organisms were obtainable in this way, cultures of the throat and urine were made by similar methods. In addition to these cultures opportunity was afforded by two autopsies to obtain growths from the tissues of the lymph glands, spleen and kidney. The methods of culture used were as follows:

Blood Cultures.—From 10 to 15 c.c. of blood were taken and cultures were made in different ways. The blood was usually divided into two parts. To one part were added 1-2 volumes of 1 per cent sodium citrate in normal salt solution. The other part was made into anaerobic plates according to the method described by Krumwiede.¹

According to this method the fresh blood was added to tubes of melted agar at 39-40 C. in the proportion of about 1 c.c. to about 9 c.c. of agar. After the blood and agar were thoroughly mixed, the mixture was poured into an inverted top of a Petri plate, and the bottom, also inverted, laid directly on the melted agar. As soon as the agar solidified, the space between the rims of the two parts of the plate and over the exposed agar was sealed with boiling paraffin. From 6 to 12 of these plates were made and incubated at 35-37 C. Plates were allowed to incubate for a month or more before they were considered sterile, but for the most part growths did not appear after two weeks. The technic of making the anaerobic plates was varied in three ways. In most cases ascites fluid was added to the agar and also bits of sterile rabbit kidney or spleen. In some cases the entire quantity of blood taken from the patient was citrated and the plates made from the citrated blood.

With citrated blood, cultures were usually made in one of two ways. The corpuscles were allowed to settle by simple gravitation at room temperature. At the end of an hour the clear citrate plasma was pipetted off and cultures made by adding the fluid to melted ascites agar in tubes with a bit of sterile rabbit kidney at the bottom. After the agar solidified, the tube was made anaerobic by adding sodium hydrate and pyrogalllic acid to the cotton plug and sealing the tube

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1. *Jour. Infect. Dis.*, 1913, 12, p. 199.

with a paraffined cork. In other cases the citrated blood was centrifugalized at about 3,000 revolutions per minute for one-half hour, and then, after being decanted and having sterile distilled water added to it, was centrifuged in the same way again. By repeating this procedure the hemoglobin was eliminated and cultures made in the same way from the sediment as from the citrated plasma.

Cultures from the organs at autopsy were made by cutting out blocks of tissue with sterile instruments, searing the surface by immersion in boiling water, and macerating in a mortar. The liquid thus obtained was spread over the surface of ascites blood agar slants and added to tubes of ascites agar with rabbit kidney and made anaerobic. Cultures from catheterized urine were made by centrifugating 25-50 c.c. of urine for one-half hour at about 3,000 revolutions per minute and making cultures from the sediment in the same way as from the blood sediment.

Subcultures were made on anaerobic ascites goat blood agar slants from everything in the plates and tubes resembling colonies. Direct smears were also made at times but it was difficult in many cases to decide from direct smear whether or not organisms were present in what appeared to be a colony, inasmuch as some organisms were small, pleomorphic, few in number, and hard to distinguish from artefacts. In the plates, growths extending in from the edges were regarded as contaminations and were not examined. As a rule there developed from the 10-15 c.c. of blood used only a few colonies.

Cultures from the throat were made by shaking off the particles from swabs of the tonsils into plain broth and making cultures from this in serial dilutions as from the fluid obtained from organs. The throat cultures were made after a number of cultures from other sources were grown, with the idea of obtaining evidence as to the source of the organisms found in other parts of the body.

A brief description of the organisms found is given in the tables. A few of the throat cultures are tabulated in order that the organisms found may be compared with those in the other tables.

The results of the examination of the spleen, lymph glands and pericardial fluid of Case 1 follow. The pericardial fluid gave in the Noguchi cultures a peculiarly shaped organism (see Fig. 1, Plate 1), which developed in five days, and in two weeks a white snowball-like colony formed. The organism was strictly anaerobic and grew only on blood or ascites media. The spleen gave (*a*) a hemolytic streptococcus, (*b*) an anaerobic pigment-forming bacillus growing in jet-black colonies, (*c*) long threadlike organisms growing only anaerobically and (*d*) diphtheroid bacilli. The cervical lymph glands gave (*a*) a hemolytic streptococcus, (*b*) a non-hemolytic streptococcus growing best anaerobically, (*c*) a fine pleomorphic Gram-negative bacillus, and (*d*) long threadlike anaerobic Gram-negative organisms. The axillary and inguinal lymph glands yielded no growths.

In Case 2 the spleen was not examined. The axillary and inguinal lymph glands were sterile; aerobic cultures of the cervical lymph glands yielded a hemolytic streptococcus.

A comparison of the organisms obtained from the blood, throat, urine and of the organs at autopsy shows that, to a great extent, the same organisms are found in all of them.

A discussion of the organisms which, on account of the frequency of their occurrence in this series are of particular interest, follows. Their pathogenic powers were tried usually on guinea-pigs, but also on dogs and rabbits from which the hair was removed with potassium sulphid in order that rashes might be observed.

1. *Small Pleomorphic Gram-Negative Organisms*. — Occasionally Gram-positive or doubtful, these organisms resemble cocci so closely at times that they are hard to distinguish from cocci without some study; they vary somewhat in size, but as a rule are about the length of an influenza bacillus though more slender; they are motile to a varying degree. In the anaerobic cultures the growth usually appears in from one to two weeks as a fine pin-point-like colony which reaches the size of a millimeter or more in diameter. On the surface of blood agar slants these organisms form delicate, transparent, pin-point colonies. After subcultures have been made for a time, the organisms grow to a varying extent aerobically and after a number of months a comparatively profuse growth may be obtained in from twenty-four to forty-eight hours, the organisms growing to some extent on plain media also. In 7 of the 20 positive cultures these organisms were found in the blood. They were isolated from the throat in pure culture in 3 cases, from the kidney in both cases examined; from the urine in 3 of 6 cases examined, and from the lymph glands and spleen at autopsy. These organisms vary in pathogenic effect, but as a rule are fatal for guinea-pigs in the small doses used. They cause death after subcutaneous injection in from twelve to twenty-four hours. In one case a localized erythema appeared a few minutes after the injection. Agglutination tests were made with all the organisms of this type which were isolated, convalescent scarlet fever serum being used, and as a control, serum from convalescent diphtheria patients. The results follow:

1-3.	No agglutination.	
E-3.	Scarlet serum 1-40	Control 1-80
O-2.	Scarlet serum 1-160	Control 0
V-1.	Scarlet serum 0	Control 0
H-2.	Scarlet serum 0	Control 0
B-1.	Scarlet serum 0	Control 0
II-2.	Scarlet serum 1-40	Control 0

TABLE I
ORGANISMS FOUND IN BLOOD

Case	Morphology	Gram	Aerobic or Anaerobic	Blood	Litmus Milk	Litmus Dextrose	Litmus Mannite	Litmus Inulin	Litmus Lactose	Plain Agar	Character of Growth
A	1. Small pleomorphic bacillus, motile	+	Anaerobic	No effect			No growth	No growth			Very small, transparent colonies
B	1. Small pleomorphic bacillus, tumbling motility	+	"	"			No growth	No growth			Small, transparent, round colonies
C	1. Small streptococcus in short chains	+	Aerobic	"	Slightly Acid	Acid	Acid	—	Acid	Fair growth	Small, round, white, opaque colonies
	2. Spore-forming bacillus	+	"	"	Good	"
	3. Small pleomorphic bacillus	+	Anaerobic	"	—	—	—	—	—	Fair	Small, transparent colonies
D	1. Diphtheroid bacillus	+	Aerobic	"	—	Acid	—	—	—	Good	Grayish, translucent film
E	1. Streptococcus in fairly long chains	+	Hemolysis
	2. Pigmented pleomorphic organism	—	Anaerobic	"	No growth	No growth	No growth	Black, convex, fairly large colonies
	3. Small pleomorphic bacillus, motile	—	"
	4. Small staphylococcus	±	No growth
F	1. Spirillum	—	Anaerobic	—	No growth	No growth	Pin-point, transparent colonies
G	1. Diplococcus	+	Aerobic	Acid	—	—	—	—	Good growth	Moist, grayish, semi-transparent film
	2. Diplostreptococcus of varying size	+	"	Brown hemolysis	Acid	Acid	Acid	Acid	Acid	"	White, moist, translucent film
	3. Small streptococcus	+	"	—	Acid	—	—	—	"	Moist, yellowish film
	4. Short, plump bacillus with rounded ends	+	"	Green hemolysis	—	Acid	"	Small, transparent colonies
	5. Spore-forming bacillus with square ends, motile	+	"	"	Coag., bleached	"	Acid	Acid	—	"	Dry, heavy, white, adherent growth
	6. Biscuit-shaped diplococcus	—	"	Slight hemolysis	—	—	—	—	—	"	Lemon-yellow, moist, opaque film
H	1. Small diplostreptococcus	+	"	Green	Acid	Acid	—	—	"	White, translucent film
	2. Spore-forming bacillus with square ends	—	"	Green Hemolysis	Pept. bleached	Bleached	Bleached	Acid	"	Heavy, white, dry, adherent film
	3. Staphylococcus	+	"	Green	Acid, coag.	Acid	—	"	Acid	Fair	Delicate, translucent film
I	1. Spore-forming bacillus with rounded ends	—	"	Hemolysis	Bleached, coag.	Gas, acid	Acid	"	—	Good	Cream-colored, opaque, moist film
	2. Oval streptococcus, rather large	+	"	"	Acid	Acid	—	—	—	Fair	Small, round, transparent colonies
	3. Staphylococcus	+	"	"	Acid, coag.	"	Acid	—	Acid	Good	Opaque, white smear
	4. Square end, spore-forming bacillus in chains	..	"	"	Peptonized	"	—	—	—	"	Thick

TABLE 1.—(Continued)

		+	Aerobic	Hemolysis	Bleached, ct. ag.	Bleached, acid	Acid	—	—	Good growth	Dry, white, opaque film, very adherent
J	1. Spore-forming bacillus	+	"	"	—	Acid	—	—	"	"	Thick, moist, opaque, dull-yellow film
	2. Short, plump bacilli with rounded ends; spores?	—	"	"	Acid	—	—	—	"	"	Opaque, white film
	3. Staphylococcus	+	"	"	—	—	—	—	Fair	"	Delicate, moist, translucent film
	4. Small bacillus, pleomorphic	—	"	No	"	Very small, transparent colonies
	5. Small streptococcus	..	"
K	1. Small coccus	+	"	Hemolysis	Bleached, acid	Acid	—	—	Good	"	Profuse growth of sharply outlined, yellow colonies
	2. Motile threadlike organism	—	Obl. anaerobic	—	—	—	—	—	Slight	"	Small, transparent colonies
L	1. Short, plump bacillus	—	Aerobic	—	—	Acid	—	—	Good	"	Moist, semitransparent film
M	1. Welch bacillus	+	Anaerobic	Hemolysis	Coag., acid	Gas, acid	—	—	Poor	"	Rather large, dewdrop-like colonies
N	1. Streptococcus	++	Aerobic	—	—	Acid	—	—	Fair	"	Transparent film
	2. Diplococcus, fairly large	+	"	—	—	"	Acid	—	Good	"	Pearly white, round colonies
O	1. Large diplococcus	+	"	Slightly acid	"	—	—	"	"	Orange-colored
	1. Spore-forming bacillus	+	"	Hemolysis	Pept., bleached	Bleached	Bleached	—	"	"	Dull-white, moist film, not adherent
P	2. Spore-forming bacillus	++	"	"	—	Acid	—	—	"	"	Translucent film
	3. Small streptococcus, short chains	+	"	"	Coag., acid	"	Acid	—	"	"	Moist, white, translucent film
	4. Slender bacillus	—	Coag., bleached
	5. Spore-forming bacillus in filaments	—	Aerobic	Hemolysis	Coag., bleached	Bleached	—	—	Good growth	"
Q	1. Large diplostreptococcus	++	"	"	"
	2. Large spore-forming bacillus	+	"	Acid	Acid	Acid	—	"	"	Wrinkled, moist film
R	1. Threadlike organism	—	Not grown in pure culture
S	1. Small pleomorphic bacillus	—	Anaerobic	—	No growth
T	1. Streptococcus	+	Aerobic	Hemolysis	Coag., acid	Acid	—	Acid	Good growth	White, gradually becoming orange
	2. Small pleomorphic bacillus	±	Aerobic and anaerobic	Slight hemolysis	—	—	—	—	—	"	Cream-colored translucent film

TABLE 2

ORGANISMS FOUND IN THROAT

Case	Morphology	Gram	Aerobic or Anaerobic	Blood	Litmus Milk	Litmus Dextrose	Litmus Mannite	Litmus Inulin	Litmus Lactose	Plain Agar	Character of Growth
O	1. Streptococcus, irregular in size, short chains	+	Aerobic and anaerobic	Hemolysis	Acid	Acid	No effect	No effect	Acid	Fair growth	Delicate, transparent
	2. Small pleomorphic bacillus, motile	-	"	No effect	No growth					Slight	" colonies
	3. Small diplostreptococcus	-	Anaerobic	"	No effect	Acid	No effect	No effect	No effect	Fair	Delicate, transparent
	4. Wavy, threadlike organism	-	"	"	"	"	"	"	"	"	No growth. Not obtained in pure culture
	5. Fusiform bacillus	-	Anaerobic	"	"	"	"	"	"	"	No growth. Not obtained in pure culture
G	1. Diplococcus	-	Aerobic and anaerobic	No effect	Acid	Acid	No effect	No effect	No effect	Fair growth	Delicate, transparent, round colonies
	2. Streptococcus in fairly long chains	+	Aerobic and anaerobic	Hemolysis	"	"	"	"	Acid	"	Delicate, transparent, round colonies
	3. Cocci occurring singly, in pairs, chains, and clusters	±	Aerobic and anaerobic	"	"	"	"	Acid	No effect	Good	Small, hemispherical sharply outlined lemon-yellow colonies
	4. Rather small streptococcus	±	Aerobic	"	No effect	"	Acid	No effect	"	"	Moderately thick, whitish film
	5. Curved threads of varying length with bacillary forms	-	Anaerobic	No effect	No growth					Slight	Small, transparent colonies
	6. Pigmented pleomorphic organism	-	"	Hemolysis	No growth						Large, convex, black to grow colonies hard to
	7. Slender bacillus with terminal spores	-	"	"	Faintly acid	Acid	Acid	"	Faintly acid	Slight growth	Slight growth except on dextrose agar
	8. Slender bacillus	-	Aerobic	No effect	No effect	No effect	No effect	No effect	No effect	Good	Fairly heavy, semi-transparent, moist film

TABLE 2—Continued

[illegible]

TABLE 3
ORGANISMS FOUND IN URINE

Case	Morphology	Gram	Aerobic or Anaerobic	Blood	Litmus Milk	Litmus Dextrose	Litmus Mannite	Litmus Inulin	Litmus Lactose	Plain Agar	Character of Growth
D	1. Somewhat long, slender bacillus resembling <i>E. tuberculosis</i>	—	Aerobic	Green	—	Bleached	—	—	—	+	Moist, somewhat wrinkled film, whitish, turning yellow later
	2. Small pleomorphic bacillus	—	Aerobic and anaerobic	"	Slight bleaching	Acid	—	—	—	—	Moist, gray, translucent film; old colonies yellow
	3. Small pleomorphic bacillus. Same as D2?	—	"	—	Slightly acid	—	—	—	Slightly acid	+	Delicate transparent growth
	4. Spore-forming bacilli with many bizarre forms	+	Aerobic	Green hemolysis	—	Bleached	—	Acid	—	+	Rather thick, almost transparent film
	5. Pleomorphic pigmented organism	—	"	"	"	"	"	"	"	"	Not obtained in pure culture
	6. Wavy, threadlike organisms	—	"	"	"	"	"	"	"	"	Not obtained in pure culture
G	1. Ordinary staphylococcus	+	Aerobic	"	Acid, not coag.	Acid	Acid	—	Acid	+	Moist, semitranslucent, grayish film
	2. Spore-forming bacillus slightly larger than <i>B. coli</i>	—	"	Brown hemolysis	Coag., bleached	"	"	Acid	—	+	Heavy, opaque wrinkled, brown film
	3. Streptococcus. Medium long chains	+	"	Hemolysis	—	"	—	—	—	+	Delicate, transparent growth
	4. Vibrio—larger and more slender than comma bacillus	—	"	"	Acid	"	—	—	—	+	Yellowish, transparent film
	5. Pleomorphic pigmented organisms	—	"	"	"	"	"	"	"	"	Not grown in pure culture

TABLE 3—Continued

N	1. Diplococcus resembling Dip. catarrhalis	—	Anaerobic	—	—	Bleached	—	+	—	Heavy white, opaque growth
	2. Streptococcus in short chains	+	Aerobic	—	—	Acid	Acid	—	Acid	+	Heavy brownish growth
	3. Diphtheroid organism	+	Not grown in pure culture
V	1. Very small pleomorphic bacillus, motile	+	anaerobic	—	No growth	Slight	Delicate, transparent growth
	2. Round diplococcus about size and shape of ordinary staphylococcus	+	"	—	Acid, coag., bleached	—	—	Acid	Good growth	Transparent film
	3. Long, wavy, threadlike organisms	—	Not grown in pure culture
	4. Pigmented bacilli, pleomorphic	—	Not grown in pure culture. Round, convex, black colonies
X	1. Very small pleomorphic bacilli	—	Not grown in pure culture
Y	1. B. coli morphology	+	Aerobic	No growth
	2. Diplococcus-gonococcus morphology	—	"	No growth
	3. Influenza-like bacilli	—	Anaerobic
Z	1. Spore-forming bacillus, slightly larger than coli	—	Aerobic	Hemolysis	—	Acid	Acid	Acid	+	Moist, luxuriant growth
	2. B. coli	—	"	—	Acid cong.	"	"	—	"	+
	3. Pigmented pleomorphic organisms	—	Anaerobic	Hemolysis	No growth	Round, convex, black colonies

TABLE 4
ORGANISMS FOUND IN KIDNEY

Case	Morphology	Gram	Aerobic or Anaerobic	Blood	Litmus Milk	Litmus Dextrose	Litmus Mannite	Litmus Inulin	Litmus Lactose	Plain Agar	Character of Growth
I	1. B. coli 2. Staphylococcus 3. Small pleomorphic bacillus, motile 4. Long, wavy, threadlike organisms	+ + - -	Aerobic " Anaerobic "	- -	Coag., acid	Acid, gas	+	-	+	+ No growth No growth
II	1. Bacillus similar to B. coli 2. Small pleomorphic bacillus, motile 3. Long, wavy, threadlike organisms 4. Very fine coccus 5. Pigmented pleomorphic organism	+ - - ± -	" " " " " - Hemolysis Coag., alk. Alk. - - Alk. Slight	No growth No growth No growth Very fine dustlike colonies

It will be seen that the results of the agglutination tests are irregular and somewhat contradictory. In two cases, however, there was more agglutination with scarlet serum than with normal serum. The test is of little significance, as the presence of the organism even as a secondary infection might result in agglutinin formation.

2. *Pigment-Forming Organisms*.—Such organisms are not infrequently described in connection with various pathologic processes, including appendicitis, lung abscess, renal infections, etc. They are also of protean morphology, for the most part, however, small bacillary forms shorter than colon bacilli. They appear in the cultures after a few days as pin-point, semi-transparent colonies which gradually absorb coloring material from blood or tissues and become a tarlike mass of jet-black pigment. They were found in the blood, the spleen and the kidney in the two autopsies; in the urine of 4 cases of 7 examined. They were also found in the throat in many more cases than are represented in the table. In the throat cultures of chronic tonsillitis, however, they were quite as common. They are toxic for guinea-pigs and cause death in from twelve to twenty-four hours, according to the dose used, but produce no lesions suggestive of scarlet fever.

3. *Long Threadlike Organisms*.—These organisms were exceedingly difficult to isolate in pure culture and the only one which was obtained in any quantity for experimental work was from the blood. It grew slowly, taking a number of days to develop; after a considerable time it grew also on Löffler's blood-serum and ordinary media. It was at first anaerobic, but later grow aerobically. After a long period of growth, however, the organism took on a morphology more like that of the diphtheria bacillus than its original threadlike form. It was very toxic for guinea-pigs and on intravenous injection caused almost immediately subcutaneous petechial hemorrhages and in a few minutes death with convulsions. The subcutaneous injection resulted in several animals in petechial rashes and death in from twelve to twenty-four hours. The organism was agglutinated by scarlet serum in a dilution of from 1 to 40 and by diphtheria serum in a dilution of from 1 to 80.

4. *Spore-Forming Bacilli Somewhat Like Bacillus Subtilis*.—This grew best aerobically, the growth being very adherent to the media. The organisms were regarded at first as contaminations, but a record was kept of their occurrence. Later, however, they were isolated from

the throat, and in the examination of direct smears from the throat organisms of this shape occur in a high percentage of cases. The injection subcutaneously of a considerable quantity of these organisms into a guinea-pig resulted in a diffuse generalized erythema in about twenty-four hours, followed a few days later by a desquamation resembling that of scarlet fever. It was accidentally found during an attempt to obtain growths from throat mucus passed through Berkefeld filters that these organisms pass through the filter much more readily than do smaller organisms. This may be due to the minute spores which were later demonstrated with ordinary stains for spores. It is difficult to interpret the animal experiments. They were repeated a number of times and rashes obtained in some cases and not in others.

5. *Hemolytic Streptococci*.—These organisms were found in the blood in 7 of the 20 positive cases, in the lymph glands in 2 cases, in the spleen in 2 cases and in the urine in 3 cases. These results are simply confirmatory of those of a large number of other observers.

6. *Very Small Streptococci*.—In 5 cases such organisms were isolated from the blood. They were much smaller than the hemolytic streptococci, did not as a rule affect blood and occurred very often in clusters as well as chains.

7. *Gram-Negative Diplococci*.—These organisms were recovered from the blood in 1 case, from the urine in 2 cases, and from the throat in 1 case. Injection of the organism from Case G-6 resulted in a rash closely resembling that of measles. At autopsy the kidneys showed a marked cloudy swelling.

8. *Large Gram-Positive Diplococci*.—These were found in the blood in 3 cases, in the urine in 2 cases and in the throat in 1 case. The pathogenic effect on animals was not tested.

9. *Diphtheroid Bacilli*.—These organisms were recovered from the blood in 1 case, from the urine in 1 case and from the throat in 2 cases. No tests were made on animals to determine whether they were true diphtheria bacilli or not.

It will be seen from the tables that a number of other organisms, including *B. welchii*, were recovered from the blood. One of the most interesting of the organisms found was the spirillum in Case F. The organism was found only in one blood-culture, but it grew in very minute colonies which were hard to find, and even had it been present in the blood in a high percentage of cases it might readily have been

overlooked. In a series of mixed cultures made by simply inoculating ascites broth from the mucus from scarlet angina, spirilla closely resembling the one recovered from the blood were found in nearly all of them. They were also found, however, in one of four controls from the throats of diphtheria cases. Cutaneous tests with killed cultures of this organism in scarlet convalescents gave no decisive results.

SUMMARY AND CONCLUSIONS

In blood-cultures from 24 cases made anaerobically, positive results were obtained in 20 cases.

A comparison of the kind of organisms found in these blood-cultures with the organisms present in the throat and urine would indicate that during the acute stage of scarlatinal angina, organisms enter the blood-stream in considerable number and are excreted in part at least in a viable condition through the kidneys.

The excretion of living bacteria through the kidney may have some connection with the development of scarlatinal nephritis.

A number of organisms are described which are commonly associated with scarlet fever, some of which produce rashes in guinea-pigs, but it is not possible to say that any of these animals really had scarlet fever.

EXPLANATION OF PLATE 1

FIG. 1.—Spirillum from pericardial fluid (Case 1). X 1200.

FIG. 2.—Spirillum from blood (F, Table 1). X 1200.

FIG. 3.—Pleomorphic bacillus from the urine (D2, Table 3). X 1200.

FIG. 4.—Pigment-forming bacillus from the throat (H2, Table 2). X 1200.

FIG. 5.—Bacillus from the blood (C3, Table 1). X 1200.

PLATE 1



Fig. 1



Fig. 2

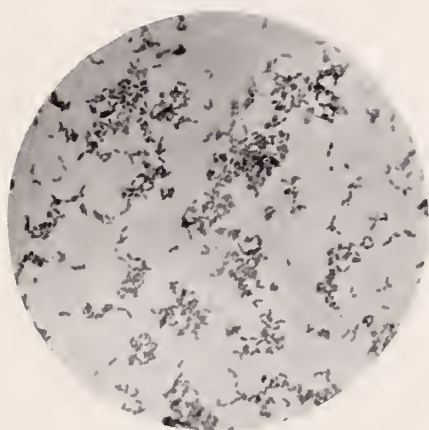


Fig. 3

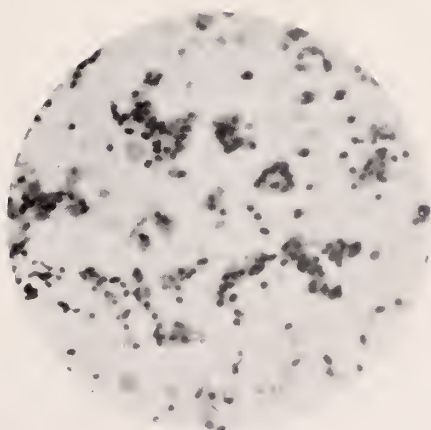


Fig. 4



Fig. 5

THE CHARACTERISTICS OF BACTERIA OF THE COLON TYPE FOUND IN BOVINE FECES*

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INTRODUCTION

In a former paper¹ from this laboratory giving the results of a study of bacteria of the colon group occurring in milk, three principal points were made: (1) The gas ratio and volume under uniform conditions are quite constant; (2) on the basis of the gas ratio and volume the collection of cultures under observation was divided into two distinct groups, one of which was probably a mixture of two or more varieties, while the other, giving a CO_2 to H_2 ratio of approximately 1.1, was distinct and homogeneous; (3) the fermentation of carbohydrates and alcohols is closely correlated with the gas ratio, indicating that this marks the lines of natural relationship within the group.

We found in the milk samples one group holding to the type with great uniformity and distinguished by the comparatively small volume of gas, a CO_2 to H_2 ratio of 1.1, and by a somewhat limited ability to ferment carbohydrates and alcohols. A considerable percentage of this group, however, fermented the resistant alcohol dulcitol. About 50 per cent. of the cultures isolated from milk gave a ratio varying from 1.5 to 2.8, a large volume of gas, and, in general, the ability to ferment all of the test substances used with the exception of dulcitol. A small number liquefied gelatin, a character apparently correlated with the fermentation of glycerin.

The low ratio group evidently included those members of the colon group usually designated as *Bacillus coli communis* and *Bacillus coli communior*, while in the high ratio group we may recognize *Bacillus lactis aërogenes*, *Bacillus acidi lactici* and possibly *Bacillus cloacae*.

In determining the sanitary quality of both water and milk, the origin of bacteria of the colon type and the directness with which they

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1. *Jour. Infect. Dis.*, 1914, 14, p. 411.

are transmitted from their natural habitat to the water or the milk are matters of great importance. In previous work the difficulty of making subdivisions of the group which were not purely arbitrary, and consequently uncertain, has prevented the determination of the sources of the colon group in more than a very general way. With more promising methods of differentiation, we were encouraged to determine the habitat of the various varieties and especially to secure more complete information on our high-ratio group. With this end in view, a representative collection of gas formers of the colon type was isolated from cow feces. These cultures were studied, as before, on the basis of the gas formation as determined by exact methods, the formation of indol, the liquefaction of gelatin and the fermentation of a set of carbohydrates and alcohols. These results are tabulated to show the correlations which may indicate the separation of the group into naturally related divisions.

ORIGIN OF THE CULTURES

The 150 cultures studied in this series were obtained from seventeen samples of feces from as many different cows on the Dairy Division farm. A small part of the sample was transferred with a platinum loop to a water blank from which lactose-gelatin and asparagin agar plates were made. This latter medium, which has been tried extensively in this laboratory by Mr. Ayers, has been found particularly adapted to the isolation of the colon group. From the two sets of plates, typical colonies were transferred to dextrose broth tubes containing small inverted tubes. From those showing gas, after incubation at 30 C., five to ten were selected and replated. The tubes were selected so that the final cultures in each case came partly from the asparagin agar plates incubated at 30 C., and partly from the gelatin plates incubated at 20 C.

MORPHOLOGY

The morphology of all cultures was determined by the examination of stained preparations made from agar cultures incubated twenty-four hours at 30 C. Camera lucida drawings were made, using a combination which gave a magnification of 4,800 diameters on the drawing board. Typical cells, selected from a number of cultures, are shown in Figure 1. These do not include the very long thread-like cells which occur occasionally. There is a wide variation in both the size and the

form of the cell, but since these variations frequently occur within the limits of a single culture they are without varietal significance.

THE LIQUEFACTION OF GELATIN

As a rule, a culture liquefying gelatin is not included in the colon group although in the report of the committee of the American Public Health Association² the classification of this group provides for strains which liquefy gelatin. In our collection from milk we found a few cultures responding to this test. The cultures under consideration were tested by adding a few drops of broth culture to the surface of a gelatin tube, paraffining the plug to reduce evaporation, and measuring the liquefaction after 30 days at 20 C. None of this collection gave any evidence of liquefaction.

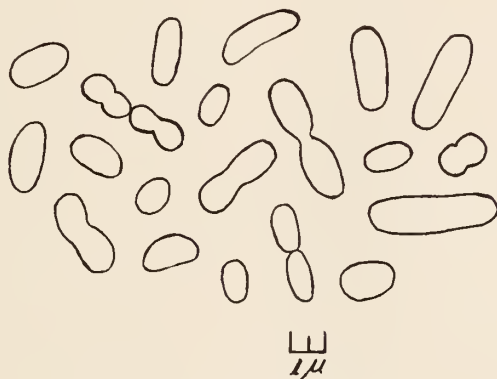


Fig. 1.—Morphology of typical cells.

THE INDOL TEST

Indol was determined by the method of Zipfel.³ The determination was made in a medium consisting of 1,000 c.c. water, 0.3 gm. of tryptophan and 5 gm. of K_2HPO_4 . Ten c.c. of this medium were used and the test made after two days' incubation at 30 C. The presence of indol was determined by adding drop by drop to each tube 1 c.c. of a 2 per cent. alcoholic solution of p-dimethylamidobenzaldehyd and concentrated hydrochloric acid. If indol was present, a violet color appeared. The medium was colorless and the reaction was sharp and clear. The results are given in Table 4. It will be noted that only

2. Standard Methods for the Examination of Water and Sewage, New York, 1912, p. 80.

3. *Centralbl. f. Bacteriol.*, Abt. 1, Orig., 1913, 67, p. 572; *Ibid.*, 67, p. 572; *Ibid.*, 1912, 63, p. 545.

one of the 150 cultures failed to give a positive reaction, and that this culture was sharply differentiated from the others by its high gas volume and ratio.

THE FERMENTATION OF CARBOHYDRATES AND ALCOHOLS;
GAS FORMATION

Methods.—In the study of the gas production of these bacteria, the method of Keyes⁴ was again used, with the modifications described by Rogers, Clark and Davis.⁵ Essentially it consists in growing the bacteria in evacuated and sealed bulbs, in collecting the gas with mercury pumps and in analyzing the gas over mercury. Instead of the Topley pumps formerly used, two automatic Sprengel pumps were employed. To keep these efficiently dry, while evacuating the larger quantity of medium employed in the present series, it was found necessary to interpose a phosphorus pentoxid bulb between the fall tube of the pump and the sulphuric acid drying tube, described in our former paper. To render the Sprengel pumps available at all times for the collection of gas, we finally abandoned the practice of making the preliminary evacuation with these, and instead used a Geryck oil pump with a proper drying chain. While the preliminary evacuation could not be carried so near to completion by this means, no difference in the results could be detected. In all the evacuations the pressure was estimated by observing the nature of the discharge from an induction coil when passed through a small Plücker tube attached to the pump. Such discharges were compared at known pressures determined with a McLeod gauge, and so we are able to state that the gas pressure indicated in the pump at the conclusion of the preliminary evacuation was well below 0.06 mm., and that the gas pressure indicated at the close of the collection of the gas was considerably below 0.03 mm. As a matter of fact, the pressure of actual gas left in the bulbs at the close of the preliminary evacuation was probably somewhat lower than that indicated above because of the sweeping effect of the water vapor. There is, however, considerable difference of opinion among various authors concerning the effectiveness of this. We therefore prefer to be conservative. Furthermore, the pressure indicated at the close of the gas collection was to us simply an indication that the evacuation had reached a point where the amount of gas retained by the medium was too small to

4. *Jour. Med. Research*, 1909, 21, p. 69.

5. *Jour. Infect. Dis.*, 1914, 14, p. 411.

measure by our methods. We fully realize how nearly impossible it is to completely remove all the gas from a liquid by evacuation.

In our former series a medium made up as follows was employed:

"To 1,000 c.c. distilled water were added 4 gm. Liebig's beef extract and 10 gm. Witte's peptone. This was heated on a steam bath for twenty minutes, filtered, and the loss of water made up. The acidity of a sample was determined by titration with tenth normal sodium hydrate, using phenolphthalein as an indicator. The whole was then neutralized with normal sodium hydrate. After five minutes' heating the medium was again filtered, its reaction determined, and if 10 c.c. required more than 0.2 c.c. tenth normal sodium hydrate it was neutralized, heated, and again filtered. There were now added 1 per cent. dextrose (Kahlbaum) and 0.5 per cent. dibasic potassium phosphate (K_2HPO_4). After the solution of these was complete the medium was filtered and its volume at room temperature made up to 1,000 c.c."

In our present series the above medium was abandoned as unnecessarily complicated. The Liebig's extract was not only unnecessary for an abundant growth of the organisms we were considering, but we had reason to believe that its introduction makes a medium which is not accurately reproduceable. Furthermore, the neutralization with alkali, which is almost certain to contain some carbonate however small an amount, introduces a slight inconstant error in the determination of carbon dioxide; and it may also produce a less accurately reproduceable initial reaction in the medium than the method of preparation we will now describe.

The medium used in the present series was made up as follows:

Ten gm. Merk's "Highest Purity" dextrose, 10 gm. Witte's peptone, and 5 gm. Kahlbaum's dibasic potassium phosphate (K_2HPO_4) were added to 800 c.c. distilled water. This mixture was heated with occasional stirring, for twenty minutes over steam. It was then filtered through a Schleicher and Schull No. 588 folded filter. The filtrate was allowed to cool to 20 C. and then made up to 1,000 c.c. The substances were weighed with reasonable accuracy on a chemical balance, and all volumetric apparatus used was accurately calibrated. As soon as prepared, the medium, in accurately measured amounts, was apportioned in the previously cleaned, dried, and sterilized bulbs. It was then fractionally sterilized in steam on three successive days.

The quantity of this medium taken for each experiment was in most cases 10 c.c., and this was held in bulbs somewhat larger than those formerly used. But the old bulbs were still available and were used with 5 c.c. of medium. When this was done the data were calculated for 10 c.c. when incorporated in the tables. The manner of inoculation, and the methods of evacuation, sealing off, collecting the gas, and the analysis of the gas, are fully described in our former paper. It

TABLE 1
GAS PRODUCTION OF BACTERIA ISOLATED FROM COW FECES

Medium: 10 c.c., 1 per cent dextrose; 1 per cent peptone; $\frac{1}{2}$ per cent. K_2HPO_4 . Incubation temperature, 30 C. Incubation period, 7 days.

Gas Collected with Mercury Pumps

Organism	Total Volume in Cubic Centimeters	Percentage of CO_2	Percentage of H_2	Percentage of Residual Gas	Ratio CO_2/H_2	Organism	Total Volume in Cubic Centimeters	Percentage of CO_2	Percentage of H_2	Percentage of Residual Gas	Ratio CO_2/H_2
22hm	12.83	51.1	47.7	1.2	1.07	22jk	13.62	51.1	48.2	0.7	1.06
22hm	15.30	52.3	48.0	...	1.09	22jl	14.17	50.3	49.2	0.5	1.02
22hu	13.83	50.5	49.2	0.3	1.03	22jm	11.04	51.3	48.7	0.0	1.05
22ho	15.98	51.2	48.3	0.5	1.06	22jn	15.07	50.8	48.6	0.6	1.04
22hp	12.44	51.5	48.5	0.0	1.06	22jo	13.51	50.0	49.5	0.5	1.01
22hq	12.96	51.5	48.5	0.0	1.06	22jp	14.18	51.7	47.6	0.7	1.09
22hr	12.60	51.0	48.7	0.3	1.05	22jq	12.56	53.7	44.9	1.4	1.20
22hs	13.82	52.1	46.5	1.4	1.12	22jr	13.59	51.1	48.3	0.6	1.06
22hs	13.85	50.2	49.0	0.8	1.02	22js	12.50	51.0	48.7	0.3	1.05
22ht	12.25	50.1	48.5	1.4	1.03	22jt	14.21	51.1	48.5	0.4	1.05
22hu	14.98	52.4	47.2	0.4	1.11	22ju	14.03	53.0	46.5	0.5	1.14
22hu	13.43	49.6	49.4	1.0	1.00	22jv	14.35	50.2	49.5	0.3	1.01
22hv	13.56	51.3	48.2	0.5	1.06	22jw	13.08	52.6	46.6	0.8	1.13
22hw	16.80	51.2	48.6	0.2	1.05	22jx	14.54	50.1	49.1	0.8	1.02
22hx	15.08	50.5	49.1	0.4	1.03	22jy	15.50	51.2	47.6	1.2	1.08
22hy	13.24	52.1	47.1	0.8	1.11	22jz	13.72	51.0	48.7	0.3	1.05
22hy	10.88	51.2	47.7	1.1	1.07	22ka	13.66	52.1	46.9	1.0	1.11
22hz	16.10	50.4	47.6	2.0	1.06	22kb	14.40	50.2	49.4	0.4	1.02
22ia	13.90	49.9	49.6	0.5	1.00	22kc	12.48	51.0	47.8	1.2	1.07
22ia	15.82	51.5	47.9	0.6	1.07	22kf	12.71	51.0	48.8	0.2	1.05
22ib	13.24	50.2	49.6	0.2	1.01	22kg	14.78	51.4	47.4	1.2	1.09
22ib	14.10	52.8	46.2	1.0	1.14	22kh	13.80	50.5	48.8	0.7	1.03
22id	12.12	50.6	48.7	0.7	1.04	22ki	12.14	50.8	48.7	0.5	1.04
22ie	13.07	51.6	47.7	0.7	1.08	22kj	14.88	53.2	46.1	0.7	1.16
22if	12.26	51.1	48.0	0.9	1.06	22kl	14.71	50.4	49.1	0.5	1.03
22ij	14.39	50.6	48.4	1.0	1.04	22km	14.09	50.6	49.1	0.3	1.03
22ik	15.13	51.0	48.4	0.6	1.06	22kn	15.32	50.3	49.4	0.3	1.02
22il	13.74	52.3	47.0	0.7	1.11	22ko	10.72	49.6	49.2	1.2	1.01
22il	14.09	51.0	48.5	0.5	1.05	22kp	14.88	51.1	48.5	0.4	1.06
22im	14.52	51.2	47.4	1.4	1.08	22kq	13.58	50.4	49.0	0.6	1.03
22im	10.92	50.6	48.9	0.5	1.04	22kr	13.01	50.5	48.7	0.8	1.04
22in	13.29	51.9	47.8	0.3	1.09	22ks	14.46	51.7	47.6	0.7	1.09
22ip	12.35	51.3	47.8	0.9	1.07	22ks	15.28	51.0	48.6	0.4	1.05
22iq	14.04	52.1	44.9	3.0	1.16	22kt	14.24	51.8	47.2	1.0	1.10
22iq	14.04	52.2	47.3	0.5	1.10	22ku	14.94	51.2	48.9	...	1.05
22ir	12.69	49.6	49.5	0.9	1.00	22kv	14.37	50.3	49.3	0.4	1.02
22ir	14.51	49.8	49.7	0.5	1.00	22kw	18.40	50.3	48.6	1.1	1.04
22is	12.13	50.6	49.1	0.3	1.03	22kw	20.40	51.2	48.2	0.6	1.06
22it	12.36	51.9	48.1	0.0	1.08	22kx	12.95	50.8	48.3	0.9	1.05
22it	12.00	50.5	47.6	1.9	1.06	22ky	14.08	50.5	49.2	0.3	1.03
22iu	14.64	51.3	48.2	0.5	1.06	22kz	14.22	51.3	48.2	0.5	1.06
22iu	11.79	49.9	48.8	1.3	1.02	22la	16.02	50.4	49.1	0.5	1.03
22iv	14.36	51.1	48.7	0.2	1.05	22lc	14.24	50.4	49.0	0.6	1.03
22iw	13.81	50.0	49.9	0.1	1.00	22ld	12.68	49.9	49.6	0.5	1.01
22iw	14.15	50.4	48.8	0.8	1.03	22le	14.58	51.9	47.3	0.8	1.10
22ix	13.69	51.4	47.3	1.3	1.09	22lf	13.87	50.1	49.7	0.2	1.01
22iy	13.76	49.5	49.3	1.2	1.00	22lf	12.79	50.7	49.0	0.3	1.03
22iy	14.60	50.8	48.5	0.7	1.05	22lg	13.79	50.5	48.8	0.7	1.03
22iz	15.27	49.8	48.3	1.9	1.03	22lh	14.75	50.2	49.3	0.5	1.02
22ja	13.54	50.7	48.7	0.6	1.04	22li	15.50	51.9	47.0	1.1	1.10
22ja	12.25	50.5	48.9	0.6	1.03	22lj	17.95	51.9	47.6	0.5	1.09
22jc	14.80	51.8	47.6	0.6	1.09	22lk	13.89	50.7	48.7	0.6	1.04
22jd	14.40	50.1	49.3	0.6	1.02	22li	14.43	50.9	48.8	0.3	1.04
22je	13.82	49.7	49.4	0.9	1.01	22lm	12.86	52.3	46.8	0.9	1.12
22je	12.96	50.5	48.5	1.0	1.04	22ln	12.58	49.2	50.2	0.6	0.98
22jf	14.11	52.5	47.1	0.4	1.12	22lo	13.23	50.5	49.5	0.0	1.02
22jf	14.39	51.3	48.0	0.7	1.07	22lp	13.72	52.8	45.6	1.6	1.16
22jg	13.46	52.6	46.7	0.7	1.13	22lp	14.33	53.1	46.3	0.6	1.15
22jh	13.99	51.1	48.2	0.7	1.06	22lp	15.96	52.1	47.0	0.9	1.11
22ji	12.85	50.9	48.1	1.0	1.06	22lq	15.02	50.2	49.1	0.7	1.02

TABLE 1—*Continued*

Organism	Total Volume in Cubic Centimeters	Percentage of CO ₂	Percentage of H ₂	Percentage of Residual Gas	Ratio CO ₂ /H ₂	Organism	Total Volume in Cubic Centimeters	Percentage of CO ₂	Percentage of H ₂	Percentage of Residual Gas	Ratio CO ₂ /H ₂
221r	15.26	52.6	47.1	0.3	1.12	22mn	18.50	51.3	48.1	0.6	1.07
221r	12.68	50.6	48.9	0.5	1.03	22mn	16.37	51.4	48.0	0.6	1.07
221s	14.87	49.5	49.1	1.4	1.01	22mo	13.46	50.5	48.8	0.7	1.03
221t	16.09	49.9	49.5	0.6	1.01	22mq	13.70	50.2	49.6	0.2	1.01
221u	14.11	50.6	49.1	0.3	1.03	22mr	16.25	51.5	47.9	0.6	1.07
221v	15.72	52.0	46.6	1.4	1.11	22ms	12.17	51.5	48.3	0.2	1.07
221w	15.54	51.9	48.0	0.1	1.08	22mt	12.94	51.2	48.7	0.1	1.05
221x	14.09	53.2	46.4	0.4	1.15	22mu	15.18	51.4	48.0	0.6	1.07
221x	15.40	50.4	49.4	0.2	1.02	22mw	12.56	50.8	48.7	0.5	1.04
221x	14.25	51.2	48.2	0.6	1.06	22my	14.30	49.8	49.2	1.0	1.01
221y	14.94	49.6	49.7	0.7	1.00	22my	15.03	50.8	48.8	0.4	1.04
221y	13.98	50.3	48.6	1.1	1.04	22mz	14.42	50.4	49.2	0.4	1.03
221z	17.10	49.7	49.7	0.6	1.00	22na	14.94	53.8	45.3	0.9	1.19
221z	15.07	49.9	49.5	0.6	1.01	22na	13.13	51.0	48.0	1.0	1.06
221z	13.19	49.7	49.7	0.6	1.00	22nb	12.80	51.2	47.7	1.1	1.08
22ma	13.10	52.4	46.4	1.2	1.13	22nc	13.02	51.4	47.8	0.8	1.08
22ma	13.73	50.8	49.2	0.0	1.03	22nd	13.14	50.7	48.9	0.4	1.04
22mb	14.93	50.8	48.5	0.7	1.05	22ne	13.10	51.2	48.1	0.7	1.07
22mc	13.83	52.4	46.8	0.8	1.12	22nf	14.66	51.0	48.6	0.4	1.05
22md	13.42	50.7	48.9	0.4	1.04	22ng	14.25	51.4	47.9	0.7	1.07
22me	14.42	52.3	46.9	0.8	1.12	22nh	14.92	53.9	45.8	0.3	1.18
22mf	16.30	51.5	47.9	0.6	1.08	22nh	17.52	52.1	47.9	0.0	1.09
22mg	15.21	50.2	49.5	0.3	1.01	22ni	12.27	49.7	48.7	1.6	1.02
22mh	13.20	51.0	48.3	0.7	1.06	22ni	13.80	51.2	48.3	0.5	1.06
22mi	15.12	52.0	47.2	0.8	1.10	22nj	14.04	51.1	48.1	0.8	1.06
22mj	14.42	50.1	49.4	0.5	1.01	22nk	14.54	51.9	47.9	0.2	1.08
22mk	13.60	52.5	45.9	1.6	1.14	22nl	14.83	50.8	49.2	0.0	1.03
22mk	14.14	52.7	46.7	0.6	1.13	22nm	15.59	51.0	48.7	0.3	1.05
22ml	15.20	50.6	48.9	0.5	1.03	22nn	13.00	50.9	48.1	1.0	1.06
22mm	13.43	51.1	48.3	0.6	1.06	22np	14.64	52.5	47.1	0.4	1.11
22mn	12.50	53.4	45.6	1.0	1.17	22nq	14.12	50.4	49.1	0.5	1.03

should be noted that all gas volumes have been reduced to dryness and to normal temperature and pressure.

Discussion of Analyses.—The analyses show a very remarkable agreement in the gas production of the organisms. The significance of this will be discussed later. A preliminary discussion of the analyses themselves will prove interesting.

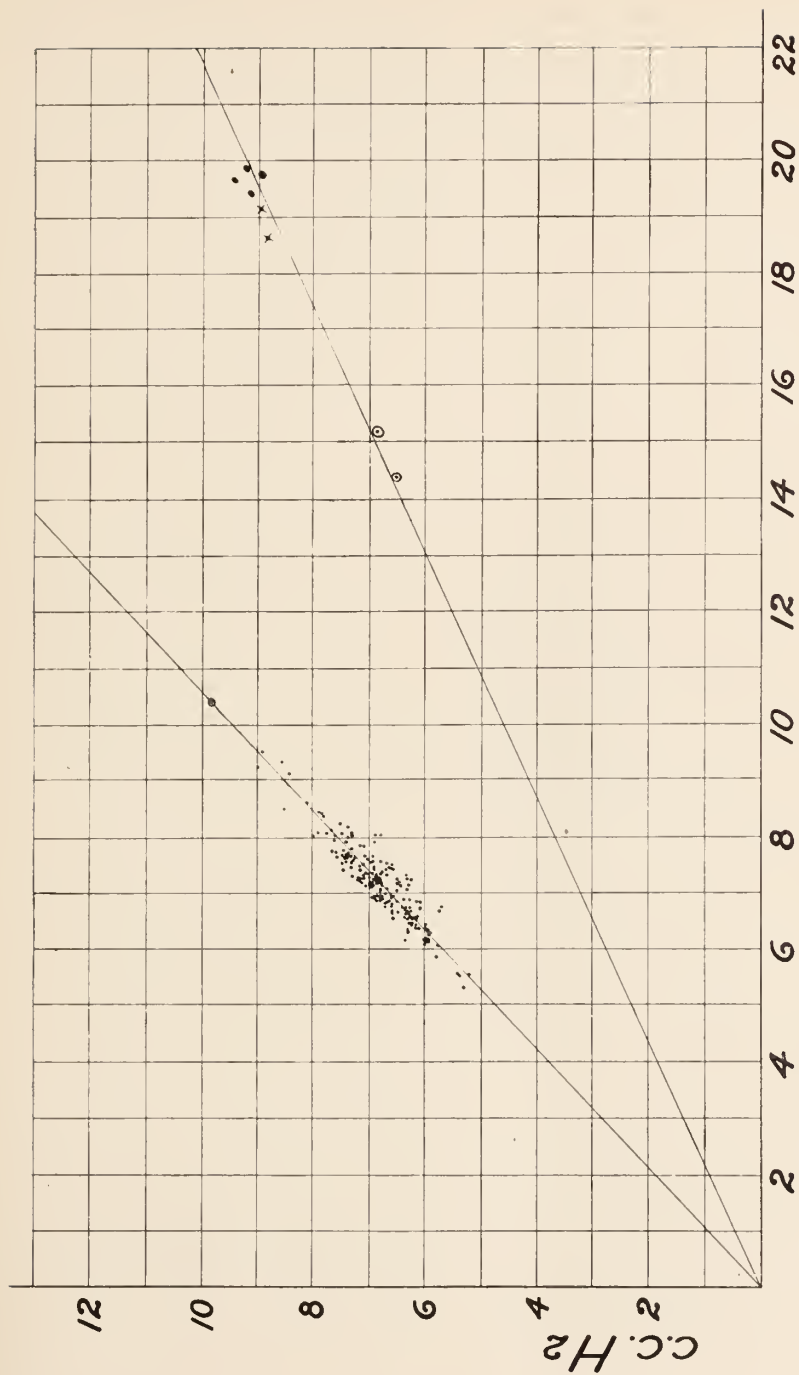
In Table 1 are assembled 182 analyses of the gas produced by 149 of the cultures isolated as described on page 100. In the first column of this table will be found the laboratory designation of the culture; in the next column, the total gas produced per 10 c.c. of medium; in the three succeeding columns the composition of this gas in percentages of CO₂, of H₂, and residual gas. The residual gas is doubtless nitrogen. No hydrocarbons have been detected. In the last column will be found the ratio CO₂ to H₂. For the experiments recorded in this table the period of incubation was 7 days, a safe but perhaps unnecessarily long period. The incubation temperature was 30 C.

As in our former series, so also in this, it was considered unnecessary to make duplicate determinations when the results of an individual analysis were close to the mean of all. But several of these determinations, which in one way or another were found to be farthest from the average, were repeated. Almost without exception this duplication placed the resultant mean distinctly closer to the total average. It should be noted, however, that this practice of repeating only the less concordant experiments makes the duplications appear less harmonious than would have been the case had all determinations been duplicated. We have excluded from Table 1 five analyses which were incomplete because of definitely known, though slight, errors in the determination of hydrogen. The percentages of carbon dioxide in these cases were 50, 49.5, 53.2, 50.6 and 51. We have also taken the liberty of excluding two analyses, one of 22kx and one of 22ht, both of which seemed to agree with their duplicates so far as they could be judged, but in both of which the data were incongruous. In one of these a leak during analysis was suspected. During the other analysis a sudden interruption occurred, and it is believed that the readings were incorrectly recorded. We have also withheld from the table the analyses of two cultures which we will discuss later.

The agreement in Table 1 is evidently sufficiently close to justify our using the arithmetical mean rather than the mode. The averages are as follows:

Total volume, 14.09; CO_2 , 51.1 per cent.; H_2 , 48.2 per cent.; residual gas, 0.7 per cent.; ratio CO_2 to H_2 , 1.06. If the average ratio, 1.06, is compared with the mode estimated for what we called the color group in our former paper, it will be seen that our present average is somewhat lower. This may be attributed in large measure to the medium. To compare the two media we picked at random five cultures of the present series and grew them in bulbs containing 10 c.c. of the old medium. The two sets of results are compared in Table 2.

These differences, though slight, are distinct and should be kept in mind when comparing the present and the old series. The nature of the agreement in Table 1 becomes clearer when the volumes of carbon dioxide and of hydrogen are plotted as they have been in Figure 2. In this figure the values of the two gases for the analyses summarized in Table 1 all fall close to the line of the equation $1.06x = y$, or in other terms $\text{CO}_2/\text{H}_2 = 1.06$; 1.06 is the average ratio of the group.



C.C. CO₂

Fig. 2.—Gas determinations on all cultures. Small dots (.) represent all cultures from feces which approximate the equation $\text{CO}_2:\text{H}_2=1.06$.
 ○ 22aj from milk; × 22ji from bovine feces.

A glance at this region visualizes the point made in the paper by Rogers, Clark and Davis,⁶ that such variations as occur in this group are essentially differences in the extent of the fermentation, differences which appear in the total volume of gas rather than in the ratio of the constituent gases. In a few instances one culture will apparently persist in giving a larger total volume than another; but whether we may legitimately draw any distinction between such cases is a question.

It is well to lay emphasis on this variation in total gas, for it is doubtless the source of considerable confusion when the gas is determined with the Smith or Durham tube. As Frierber⁷ has ably shown, two cultures which differ only in the amount of gas produced but not in

TABLE 2
COMPARISON OF THE GAS PRODUCTION OF SEVERAL BACTERIA WHEN GROWN IN VACUO ON
THE OLD AND ON THE NEW MEDIA

Medium	Organism	Total Gas in Cubic Centimeters	Percentage of CO ₂	Percentage of H ₂	Percentage of N ₂	Ratio CO ₂ /H ₂
New	22hz	16.10	50.4	47.6	2.0	1.06
Old	22hz	16.84	54.5	45.0	0.5	1.21
New	22kl	14.71	50.4	49.1	0.5	1.03
Old	22kl	17.87	53.4	46.2	0.4	1.16
New	22lw	15.54	51.9	48.0	0.1	1.08
Old	22lw	16.23	53.6	45.7	0.7	1.17
New	22lx	14.58	51.6	48.0	0.4	1.08*
Old	22lx	16.89	54.3	45.2	0.5	1.20
New	22mz	14.42	50.4	49.2	0.4	1.03
Old	22mz	16.81	53.7	45.7	0.6	1.17

* Averages.

the ratio of the constituent gases, will give totally different ratios when these are determined by any method which does not correct for the dissolved CO₂. Frierber was unfortunately not aware of the work of Keyes and Gillespie⁸ in which it was shown that the presence of oxygen alters the ratio to a large degree and from a cause not yet fully determined. In view of this fact and the other sources of error in the old methods of making the gas test, it would be futile to attempt any estimation of the error. Nevertheless, we suspect that several of our organisms which gave the larger volumes of gas would not have been identified with *Bacillus coli* had they been in the hands of those who place confidence in the ordinary methods of analysis, and who rely on a low ratio of CO₂ to H₂ as characteristic of *Bacillus coli communis*.

6. *Jour. Infect. Dis.*, 1914, 14, p. 411.

7. *Centralbl. f. Bakteriologie*, Abt. 1, Orig., 1913, 69, p. 437.

8. *Jour. Biol. Chem.*, 1912, 13, p. 305.

Among the organisms isolated by the method described on page 100 are two, the gas determinations of which demand special comment.

Early in the series 22ic gave the following data: Total gas, 22.53; CO_2 , 68.5 per cent.; H_2 , 31.1 per cent.; residual gas, 0.4 per cent.; ratio CO_2/H_2 , 2.2. This placed ic distinctly in that second group of gas producers described in our former paper. When later in the work duplicate determinations were made, ic surprised us by giving the total gas and ratio which are typical of the low-ratio group. Since only one such abrupt and distinct change has been noted in some one thousand analyses which we have made in the past three years, and since the former case was traced to a mistake in lettering the culture,⁹ a similar mistake was suspected in this instance. We therefore at once looked over the stock cultures and found that one of two tubes containing the ii culture had been labeled with no dot over the second i and might easily have been taken for ic. We were at the first prejudiced against this explanation, because, without consulting the records, we supposed that ii had been found to produce no growth in our anaërobic bulbs. In this our records showed us to be mistaken, and when ii came to be tried it was found to give the volume and ratio observed with what had been supposed to be a culture of ic. These characteristics were given continuously by ii, while ic continued to give the lower volume and ratio characteristic of the *Bacillus coli* group. We were unfortunate in this instance in that we had no dried sample of the original cultures with which to check our results, as we had in the case of cw, recorded in our former paper. But it is certain that the cultures of ic and ii had not been mixed or contaminated, since repetition of their fermentations and the observance of their cultural characteristics identified the cultures of each as the same organism originally isolated. We are therefore confident that an undotted i was read as a c. This conclusion was proposed and agreed on as possible by three of us at a time when we were prejudiced against it by the false supposition that ii gave no gas.

We therefore feel justified in taking the later data as characteristic of each of these cultures, and in saying that ii is the one and only culture among the 150 isolated as described on page 100, which gives a gas ratio distinct from those shown in Table 1. In Table 3 are summarized all the analyses made of the gas produced by ic and ii. These analyses of ii place it quite distinctly in that second group of gas pro-

9. *Jour. Infect. Dis.*, 1914, 14, p. 411.

ducers described in our former paper. We have shown the position in Figure 2 of the above values for CO_2 and H_2 obtained with ii by the four dots in the region $\text{CO}_2 = 19-20$, $\text{H}_2 = 9-10$. Near this are two determinations with 22s, an organism which fell in the second group of our former series. These are indicated by crossed dots. Closer to the origin are two circled dots indicating the position of 22aj, an organism which, in the former series on the more complex medium, fell in most but not all cases beyond 22s and in what we described as a provisional third group.

It is interesting to note that all three of these organisms give data which fall rather close to the line of the equation CO_2 to $\text{H}_2 = 2.17$ in which 2.17 is the average ratio of the eight determinations.

On these facts further comment will be made in a future paper; but at this point we wish to call attention to a remark made in our

TABLE 3
ANALYSES OF GAS PRODUCED BY IC AND II

Organism	Total Gas in Cubic Centimeters	Percentage CO_2	Percentage H_2	Percentage of Residual Gas	Ratio CO_2/H_2
ic?	22.53	68.5	31.1	0.4	2.20
ic	14.92	52.7	46.7	0.6	1.13
ic	15.58	52.7	46.8	0.5	1.13
ic	16.55	51.7	47.7	0.6	1.08
ic	15.57	51.0	48.7	0.3	1.05
ii	29.10	67.5	32.1	0.4	2.10
ii	28.65	67.8	31.7	0.5	2.14
ii	29.08	68.4	31.4	0.2	2.18
ii	28.70	68.8	30.9	0.3	2.23

former paper to the effect that we judged the discrepancies then found were due more to inaccuracies of method than to variations in the physiologic functions of the cultures. It certainly is true that there is closer agreement among the present data of the colon group, and while we should be cautious in drawing conclusions from the few determinations made with the high-ratio organisms, the eight harmonize in a remarkable way. We are inclined to attribute these facts to the simplification of the medium.

ACID FORMATION

Determinations were made of the amount of acid formed by all cultures in broth containing dextrose, saccharose, lactose, raffinose, starch, inulin, mannite, glycerin, adonite and dulcite. The broth was

made according to the following formula: Water, 100 gm.; peptone, 1 gm.; beef extract, 4 gm.; dibasic sodium phosphate, 0.5 gm.; test substance, 1 gm.

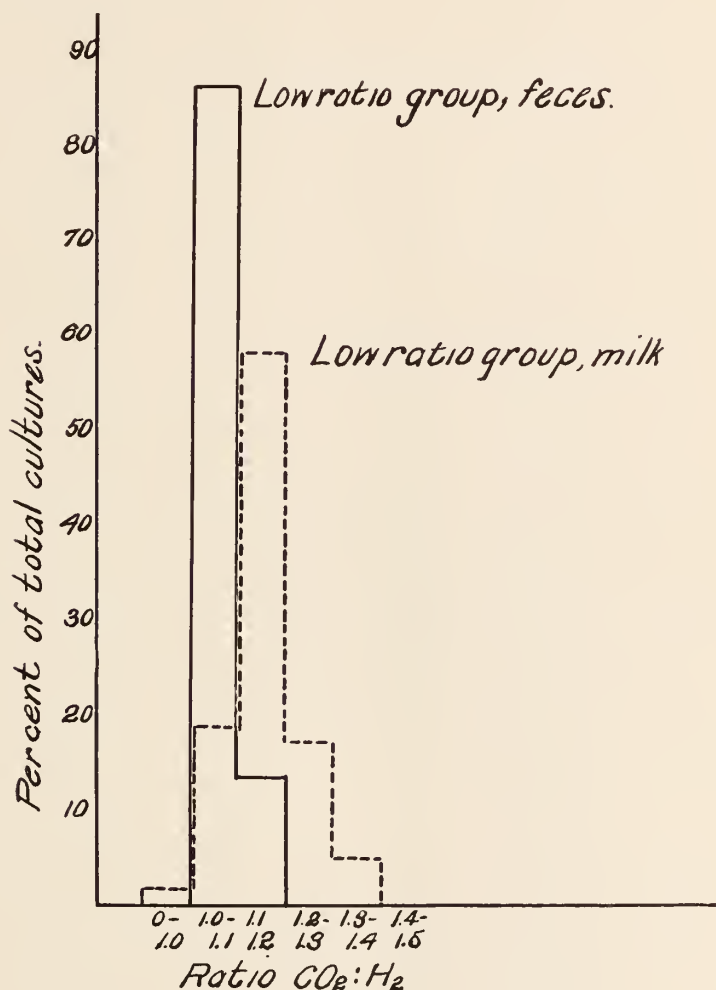


Fig. 3.—Frequency of occurrence of $\text{CO}_2:\text{H}_2$ ratios.

This was sterilized in the Arnold on three successive days. The cultures were incubated seven days and after they were steamed to remove the excess of carbon dioxide they were titrated against $n/20$ normal sodium hydrate. The results, which are given in Table 4, are

TABLE 4

FERMENTATIVE REACTIONS FOR ALL CULTURES, ACIDITY EXPRESSED AS PER CENT NORMAL ACID

Culture	Indol	Dextrose	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Adonite	Dulcite
hm	++	6.51	4.65	4.98	5.40	.00	Alk	4.75	1.30	Alk	2.30
hn	++	5.63	4.73	5.00	5.05	Alk	Alk	5.13	2.85	Alk	2.75
ho	++	5.58	Alk	5.30	Alk	Alk	Alk	4.95	2.60	Alk	Alk
hp	++	5.88	4.63	5.08	5.05	Alk	Alk	3.85	3.60	Alk	.25
hq	++	5.35	4.73	4.55	4.75	Alk	Alk	4.75	3.15	Alk	1.50
hr	++	5.52	4.75	5.40	4.95	Alk	Alk	5.05	3.60	Alk	1.10
hs	++	5.23	5.20	5.10	4.83	1.88	Alk	4.55	3.45	Alk	1.75
ht	++	5.76	Alk	5.00	Alk	Alk	Alk	4.65	3.40	1.85	Alk
hu	++	5.61	3.70	5.55	5.20	.76	Alk	4.75	3.10	Alk	3.20
hv	++	4.88	4.57	4.63	5.50	Alk	Alk	4.80	3.10	Alk	3.50
hw	++	2.63	Alk	4.85	Alk	Alk	Alk	4.85	1.66	1.80	Alk
hx	++	5.28	.85	4.60	5.05	Alk	Alk	4.45	3.40	Alk	1.50
hy	++	5.66	4.20	5.20	4.60	Alk	Alk	4.93	3.75	Alk	1.75
hz	++	5.58	4.25	4.75	5.25	Alk	Alk	4.80	3.05	Alk	3.00
ia	++	5.45	Alk	5.07	Alk	Alk	Alk	4.50	4.25	1.85	Alk
ib	++	4.91	4.68	4.40	5.25	Alk	Alk	4.85	2.45	Alk	2.75
ic	++	5.28	5.60	5.20	5.48	Alk	Alk	4.37	1.50	2.10	Alk
id	++	5.63	4.78	5.10	6.35	.90	Alk	4.52	3.00	Alk	2.30
ie	++	5.68	3.80	4.93	4.34	Alk	Alk	5.20	3.25	Alk	.70
if	++	8.78	3.70	4.95	3.70	Alk	Alk	4.75	2.70	Alk	1.75
ii	—	3.60	3.35	5.80	4.12	2.05	Alk	2.50	.05	1.40	1.40
ij	++	5.68	4.20	5.30	4.45	Alk	Alk	4.67	4.55	Alk	1.65
ik	++	5.51	5.00	4.97	5.17	Alk	Alk	4.45	2.95	Alk	3.50
il	++	5.44	4.13	5.15	4.00	Alk	Alk	4.65	2.35	Alk	3.05
im	++	5.98	4.18	5.15	4.92	Alk	Alk	4.75	3.70	Alk	2.00
in	++	5.93	4.25	4.35	4.75	Alk	Alk	4.85	3.10	Alk	1.30
ip	++	5.58	4.15	5.10	5.28	Alk	Alk	4.85	3.80	Alk	1.45
iq	++	5.60	5.47	5.22	4.85	Alk	Alk	5.13	1.90	Alk	1.50
ir	++	6.00	4.64	5.40	5.58	Alk	Alk	4.85	2.65	Alk	3.05
is	++	4.43	4.00	4.55	5.16	Alk	Alk	4.95	.80	Alk	2.60
it	++	5.73	4.15	5.80	5.47	Alk	Alk	4.70	.60	Alk	.65
iu	++	5.50	3.90	4.95	5.15	Alk	Alk	4.58	3.25	Alk	2.00
iv	++	5.73	Alk	4.83	Alk	1.03	Alk	4.73	1.80	2.25	Alk
iw	++	5.65	Alk	4.50	Alk	Alk	Alk	4.30	3.40	1.60	Alk
ix	++	5.58	Alk	5.03	Alk	Alk	Alk	4.95	1.40	1.80	Alk
iy	++	5.43	4.90	3.70	5.35	Alk	Alk	5.17	2.60	Alk	3.32
iz	++	5.73	4.70	5.90	5.00	Alk	Alk	5.05	1.15	Alk	2.70
ja	++	4.58	Alk	4.15	Alk	Alk	Alk	4.75	2.95	1.50	Alk
jb	++	5.63	Alk	5.15	.88	1.08	Alk	4.06	3.00	1.60	Alk
jc	++	5.88	5.00	5.35	5.50	Alk	Alk	4.55	2.55	Alk	2.15
jd	++	5.65	Alk	5.00	Alk	Alk	Alk	4.80	3.10	1.40	Alk
je	++	5.19	4.60	5.05	6.05	.93	Alk	4.85	1.25	Alk	2.15
jf	++	5.48	4.15	4.65	4.70	3.43	Alk	4.50	3.25	Alk	3.15
jg	++	5.66	4.25	5.05	5.45	4.75	Alk	4.60	2.15	Alk	2.35
jh	++	5.58	Alk	4.50	1.25	Alk	Alk	4.85	3.35	Alk	1.20
ji	++	5.58	4.10	5.45	5.15	3.23	Alk	4.55	3.25	Alk	3.45
jk	++	5.81	Alk	5.55	Alk	2.33	Alk	4.65	3.65	Alk	2.90
jl	++	5.73	Alk	5.05	Alk	2.88	Alk	4.70	3.35	1.95	Alk
jm	++	5.96	5.10	5.45	5.20	1.78	Alk	4.55	3.55	Alk	2.45
jn	++	5.38	Alk	2.45	Alk	Alk	Alk	5.10	2.85	1.50	Alk
jo	++	5.71	5.65	5.25	5.45	Alk	Alk	4.90	.85	Alk	2.40
jp	++	5.88	5.00	5.60	5.75	Alk	Alk	4.95	3.25	Alk	1.70
jq	++	8.08	4.00	5.40	5.70	Alk	Alk	5.00	2.20	Alk	2.70
jr	++	5.76	4.35	4.95	5.65	Alk	Alk	5.05	3.25	Alk	2.05
js	++	5.88	Alk	4.70	Alk	Alk	Alk	4.45	3.15	Alk	2.15
jt	++	5.78	3.95	5.00	5.05	Alk	Alk	4.45	3.85	Alk	2.25
ju	++	5.68	4.95	5.15	5.60	Alk	Alk	4.60	3.45	Alk	2.45
jv	++	5.53	4.35	5.40	5.80	Alk	Alk	5.10	4.15	Alk	3.20
jw	++	5.76	4.05	5.20	5.60	Alk	Alk	4.45	4.55	Alk	1.25
jx	++	5.38	4.30	5.30	5.65	Alk	Alk	3.95	2.95	Alk	1.90
yy	++	5.83	5.15	5.40	5.40	Alk	Alk	5.10	2.95	Alk	2.85
jz	++	5.58	Alk	5.25	Alk	1.68	Alk	4.80	3.15	1.80	Alk
ka	+	5.28	Alk	5.30	Alk	Alk	Alk	5.60	3.15	2.20	Alk

TABLE 4—Continued

Culture	Indol	Dextrose	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Adonite	Dulcete
kb	++	5.90	4.50	4.95	5.60	Alk	Alk	4.90	3.75	Alk	3.05
kc	++	5.83	4.40	5.15	5.60	Alk	Alk	4.80	3.65	Alk	.60
kf	++	6.08	3.10	5.10	5.50	Alk	Alk	4.80	2.75	Alk	1.15
kg	++	5.63	Alk	5.05	Alk	Alk	Alk	4.80	3.05	1.75	Alk
kh	++	5.54	4.20	5.80	5.45	Alk	Alk	4.75	1.55	Alk	Alk
ki	++	6.08	Alk	5.20	Alk	Alk	Alk	4.80	3.25	Alk	2.75
kj	++	5.73	Alk	5.30	Alk	Alk	Alk	4.80	2.95	Alk	2.95
kl	++	5.43	Alk	5.25	Alk	Alk	Alk	4.50	3.45	1.70	Alk
km	++	5.53	Alk	5.70	Alk	.58	Alk	4.80	1.35	Alk	3.00
kn	++	5.58	Alk	5.40	Alk	Alk	Alk	4.65	2.75	2.25	Alk
ko	++	6.18	Alk	4.85	Alk	Alk	Alk	5.50	4.15	Alk	3.95
kp	++	5.58	Alk	5.30	1.05	Alk	Alk	5.00	2.05	Alk	Alk
kq	++	5.08	Alk	5.00	Alk	Alk	Alk	4.60	2.35	Alk	2.95
kr	++	5.63	4.55	5.40	5.40	Alk	Alk	5.05	3.25	Alk	3.75
ks	++	5.58	Alk	4.80	.00	Alk	Alk	5.15	2.15	Alk	2.80
kt	++	5.83	Alk	5.05	Alk	Alk	Alk	4.85	3.05	2.55	3.85
ku	++	5.10	3.90	4.30	4.80	Alk	Alk	4.40	2.50	Alk	3.50
kv	++	6.00	Alk	6.30	Alk	Alk	Alk	4.95	2.95	Alk	Alk
kw	++	5.38	3.60	5.50	4.15	Alk	Alk	2.80	3.25	Alk	1.50
kx	++	5.08	Alk	5.30	Alk	Alk	Alk	3.90	3.35	Alk	2.95
ky	++	5.85	Alk	4.77	Alk	Alk	Alk	4.95	2.15	Alk	1.20
kz	++	5.63	Alk	4.75	2.80	Alk	Alk	4.55	3.25	Alk	2.30
la	++	5.70	Alk	4.15	Alk	Alk	Alk	5.00	3.65	1.70	Alk
lc	++	5.43	4.80	5.75	5.70	Alk	Alk	5.10	4.25	Alk	3.70
ld	++	5.58	4.26	5.35	5.10	Alk	Alk	4.95	2.95	Alk	1.75
le	++	5.76	4.15	5.15	4.90	4.33	Alk	4.40	4.05	Alk	3.45
lf	++	5.68	Alk	5.00	Alk	3.18	Alk	4.90	3.15	1.65	Alk
lg	++	5.58	4.30	5.90	4.90	Alk	Alk	4.15	2.70	Alk	2.80
lh	++	5.68	3.90	3.45	6.15	Alk	Alk	5.30	4.05	Alk	3.40
li	++	5.55	3.80	5.30	4.80	Alk	Alk	4.00	3.55	Alk	3.50
lj	++	5.48	Alk	2.95	Alk	Alk	Alk	4.20	2.45	1.60	Alk
lk	++	5.68	2.90	6.50	4.40	3.25	Alk	4.70	3.30	Alk	3.50
ll	++	5.15	4.10	5.65	5.35	Alk	Alk	4.90	4.05	Alk	3.30
lm	++	5.18	4.60	5.30	5.25	Alk	Alk	4.75	3.35	Alk	2.60
ln	++	5.66	3.45	5.35	5.85	Alk	Alk	4.65	4.05	Alk	2.15
lo	++	4.28	Alk	5.10	.55	Alk	Alk	3.50	1.75	Alk	.60
lp	++	5.68	3.70	5.30	4.80	Alk	Alk	4.40	3.40	Alk	2.55
lq	++	5.38	4.15	5.65	4.85	Alk	Alk	4.85	2.75	Alk	3.45
lr	++	5.55	Alk	5.60	Alk	Alk	Alk	4.55	2.05	1.90	Alk
ls	++	5.58	Alk	5.15	Alk	Alk	Alk	4.60	2.75	2.25	Alk
lt	++	5.40	3.70	5.50	5.15	Alk	Alk	4.60	3.65	Alk	4.00
lu	++	5.68	Alk	4.80	Alk	Alk	Alk	4.60	2.95	1.60	Alk
lv	++	5.31	4.45	5.40	5.15	Alk	Alk	4.65	3.15	Alk	3.85
lw	++	5.63	Alk	5.05	Alk	.38	Alk	4.60	3.75	1.60	Alk
lx	++	3.88	Alk	5.80	Alk	Alk	Alk	3.90	1.45	Alk	Alk
ly	++	6.03	Alk	5.50	Alk	Alk	Alk	4.95	1.95	Alk	Alk
lz	++	5.08	4.00	6.50	4.15	.30	Alk	4.60	2.90	Alk	2.75
ma	++	5.73	Alk	4.70	Alk	Alk	Alk	5.05	3.65	Alk	Alk
mb	++	4.98	Alk	4.80	Alk	Alk	Alk	4.40	3.25	Alk	2.65
mc	++	5.33	Alk	5.20	Alk	.83	Alk	4.60	3.55	Alk	2.60
md	++	6.78	Alk	4.80	Alk	Alk	Alk	3.90	3.00	1.85	Alk
me	++	5.73	Alk	4.55	Alk	Alk	Alk	5.15	2.95	Alk	2.80
mf	++	5.83	3.85	5.20	5.85	Alk	Alk	4.50	3.45	Alk	2.15
mg	++	5.68	4.55	4.75	5.65	Alk	Alk	4.00	2.05	Alk	3.20
mh	++	5.65	0.10	4.35	0.20	Alk	Alk	4.60	1.75	Alk	2.10
mi	++	5.78	3.50	5.20	4.35	Alk	Alk	4.20	3.00	Alk	2.45
mj	++	5.63	Alk	5.00	Alk	Alk	Alk	4.85	3.25	1.75	Alk
mk	++	6.08	3.90	3.90	4.80	Alk	Alk	4.20	3.55	Alk	2.50
ml	++	5.63	Alk	4.75	Alk	Alk	Alk	4.95	2.25	Alk	2.30
mm	++	5.88	3.70	5.60	3.65	Alk	Alk	2.70	3.40	Alk	1.70
mn	++	5.83	4.05	5.25	5.25	1.23	Alk	4.70	3.05	Alk	1.55
mo	++	5.76	3.60	5.30	4.85	Alk	Alk	3.90	3.85	Alk	3.00
mq	++	5.68	2.40	5.20	4.60	Alk	Alk	4.70	3.05	Alk	3.85
mr	++	5.58	Alk	4.30	Alk	Alk	Alk	2.80	2.30	1.40	Alk
ms	++	5.48	4.00	5.20	5.55	Alk	Alk	4.70	4.35	Alk	1.80
mt	++	5.78	Alk	4.75	Alk	Alk	Alk	5.35	3.65	Alk	Alk
mu	++	5.71	Alk	3.25	Alk	Alk	Alk	3.90	2.25	1.65	Alk

TABLE 4—Continued

Culture	Indol	Dextrose	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Adonite	Dulcite
mw	+	5.68	4.20	5.20	5.35	Alk	Alk	4.95	3.05	Alk	2.70
my	++	5.43	4.90	5.45	5.05	Alk	Alk	5.00	3.50	Alk	2.80
mz	++	7.88	3.50	6.00	5.90	Alk	Alk	3.80	2.85	Alk	3.35
na	++	6.78	4.25	5.40	5.65	Alk	Alk	4.85	3.65	Alk	.95
nb	++	5.68	3.70	5.50	5.15	1.23	Alk	4.45	4.25	Alk	2.75
nc	++	6.53	3.70	5.15	4.95	Alk	Alk	4.70	3.25	Alk	2.50
nd	++	5.58	0.15	5.05	0.35	Alk	Alk	4.10	3.25	Alk	2.80
ne	++	5.58	4.25	5.20	5.30	Alk	Alk	5.55	3.95	Alk	.40
nf	++	5.58	4.80	5.50	5.40	Alk	Alk	4.45	3.05	Alk	Alk
ng	++	5.68	Alk	5.20	.60	Alk	Alk	5.10	3.95	Alk	2.65
nh	++	5.73	3.75	4.90	5.25	0.63	Alk	6.05	3.15	1.55	2.05
ni	++	5.68	0.20	5.00	Alk	Alk	Alk	4.40	2.85	Alk	2.48
nj	++	5.50	Alk	5.15	Alk	.93	Alk	4.30	4.05	Alk	2.65
nk	++	6.08	Alk	6.30	Alk	Alk	Alk	3.30	2.90	Alk	1.00
nl	++	5.58	5.10	5.05	5.20	Alk	Alk	4.55	1.15	Alk	2.10
nm	++	5.88	Alk	5.20	1.20	Alk	Alk	4.70	2.70	Alk	2.70
nn	+	5.83	4.20	5.80	5.35	Alk	Alk	4.85	3.15	Alk	.90
np	+	5.88	4.45	5.35	5.20	Alk	Alk	4.55	2.05	Alk	.00
nq	+	6.25	Alk	6.30	Alk	Alk	Alk	4.65	2.50	Alk	Alk

expressed as percentage of normal acid. There was only a small variation in the acidity produced by individual cultures in the different test substances. The acidity is used, therefore, only as a means of determining the ability of the culture to ferment the sugar. Any reaction of 1 per cent. is considered positive.

CORRELATION OF RESULTS

In our earlier paper the marked correlation between the gas ratio of 1.1 to 1.3 and certain fermentative reactions was pointed out. The close agreement of the $\text{CO}_2 : \text{H}_2$ ratio of the feces cultures with that of low-ratio group of the milk collection has already been indicated. If the ratios are plotted on the frequency of occurrence basis, as given in Table 5, Figure 3 is obtained, which brings out very clearly the great uniformity in the results from such a large collection. For comparison, there is plotted on the same figure the milk culture frequency curve, recalculated on the basis of percentage of total low-ratio cultures. Making allowance for the difference due to the more complicated medium used with the earlier collection, we find that, so far as the gas ratio shows, the two sets of cultures are practically identical. Table 6 shows that while the amount of gas produced is subject to more variation than the gas ratio, the variation is confined to comparatively narrow limits, and when accurately determined the volume of gas from a

given amount of standard medium may be taken as characteristic of a type. In Table 7 is arranged the percentage of the total number of cultures which ferment the various test substances. In this table and those which follow the one culture giving a high ratio is excluded. In order to make these results more comprehensible they are plotted in Figure 4. By this arrangement the percentage of cultures giving a

TABLE 5
DISTRIBUTION OF CULTURES ON BASIS OF GAS RATIO

Gas Ratio	0.9-1.0	1.0-1.1	1.1-1.2	1.2-1.3	1.3-1.4
No. of cultures	6	126	29	1	0
Percentage of cultures	0	85.7	13.6	0.7	0

TABLE 6
DISTRIBUTION OF CULTURES ON BASIS OF VOLUME OF GAS

Cubic Centimeters of Gas	10-11	11-12	12-13	13-14	14-15	15-16	16-17	17-18	18-19	19-20	20-21
No. of cultures	0	1	27	40	50	20	6	1	1	1	0
Percentage of cultures..	0	0.7	18.4	27.2	34.0	13.6	4.1	0.7	0.7	0.7	0

TABLE 7
PHYSIOLOGICAL REACTIONS OF ALL CULTURES
(Percentage of Positive and Negative Reactions)

	Gelatin	Indol	Dextrose	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Adonite	Dulcitol
No. of positive reactions.	0	148	149	88	149	93	16	0	149	145	31	102
Percentage of positive reactions	00	99.3	100	59.1	100	62.4	10.7	00	100	97.3	20.8	68.2
No. of negative reactions	149	1	0	61	0	56	133	149	0	4	118	47
Percentage of negative reactions	100	0.7	00	40.9	00	37.6	89.3	100	00	2.7	79.2	31.8

positive reaction are placed on the left of the median line, while those giving a negative reaction are on the right. This shows that the type culture always forms indol from tryptophan, fails to liquefy gelatin, ferments dextrose, lactose, mannite and glycerin, frequently ferments saccharose, raffinose and dulcitol, but only rarely ferments starch, inulin or adonite. The lack of uniformity in these reactions suggests that by further subdivision, varieties would be found in which the percentage of cultures which give either a positive or negative reaction with cer-

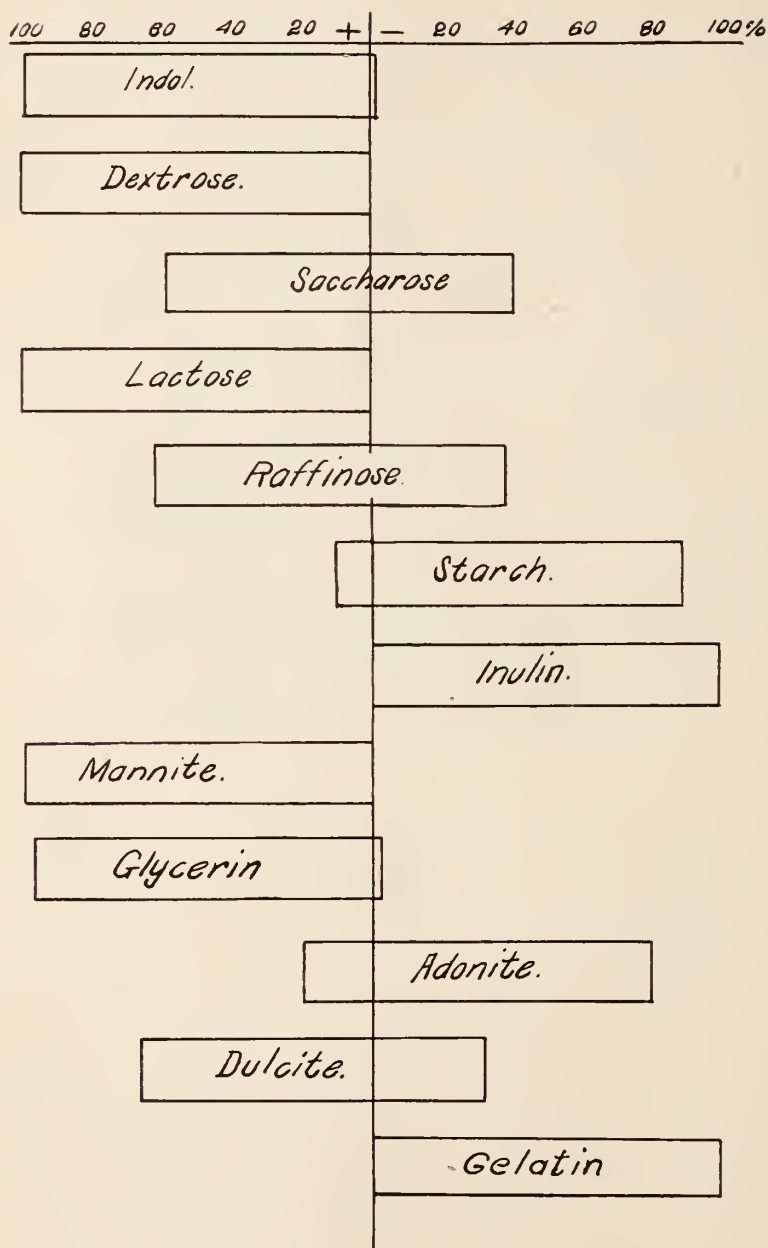


Fig. 4.—Showing ability to act on test substances. Positive results plotted to left of median line, negative results to right.

tain sugars would be materially reduced. A group answering these requirements may be obtained by dividing these cultures on the basis of saccharose fermentations. This gives us the two sharply defined groups shown in Table 8 and Figure 5. There is a positive correlation between the fermentation of saccharose, raffinose and dulcite, and a negative correlation between saccharose and adonite. This correlation between saccharose and raffinose fermentation has already been noted by Howe¹⁰ in his studies of the colon-like organisms isolated from human feces. On the one hand, we have a group the members of which ferment dextrose, saccharose, lactose, raffinose, mannite, glycerin and usually dulcite, while they almost invariably fail to utilize starch, inulin

TABLE 8
CORRELATION BETWEEN FERMENTATION OF SACCHAROSE AND OTHER TEST SUBSTANCES

	Dextrose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Adonite	Dulcite
Saccharose + (88)—									
No. of positive reactions	88	88	88	10	0	88	84	3	77
Percentage of positive reactions	100	100	100	11.4	00	100	95.4	3.4	87.5
No. of negative reactions	0	0	0	78	88	0	4	85	11
Percentage of negative reactions	00	00	00	88.6	100	00	4.6	96.6	12.5
Saccharose — (61)—									
No. of positive reactions	61	61	5	6	0	61	61	28	25
Percentage of positive reactions	100	100	8.2	9.8	00	100	100	45.9	41.0
No. of negative reactions	0	0	56	55	61	0	0	33	36
Percentage of negative reactions	00	00	91.8	90.2	100	00	00	54.1	59.0

or adonite. On the other hand, we have a group fermenting dextrose, lactose, mannite and glycerin, but which almost always fails to ferment saccharose, raffinose, starch and inulin, but not infrequently ferments adonite and dulcite.

The collection obtained from milk came from a wide range of sources and could not be expected to give so uniform results as we have obtained from the cultures coming from a single source. The striking agreement, however, which is obtained when these two collections are classified on the same basis is shown in Figure 5, and is

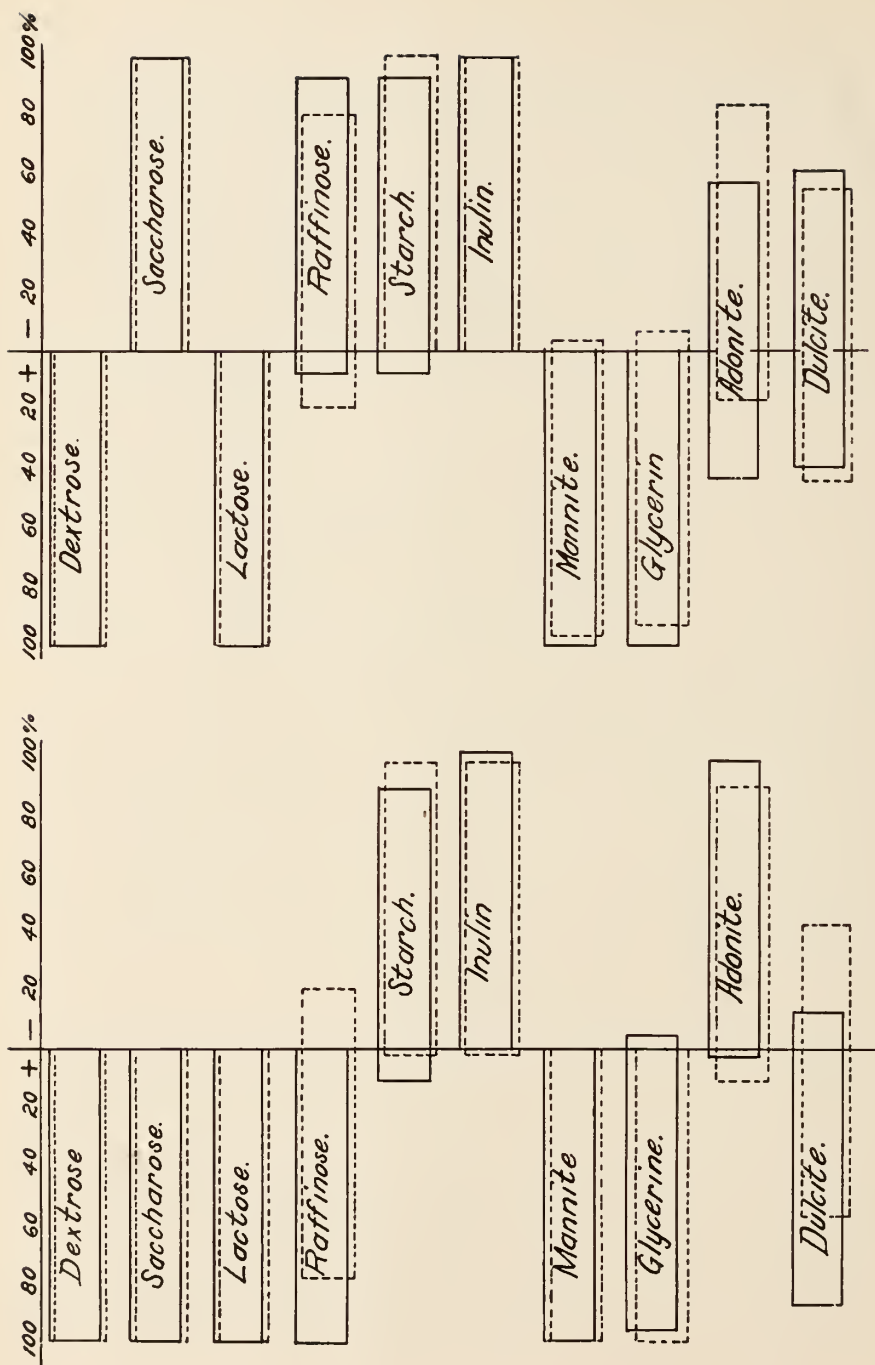


Fig. 5.—Two subgroups of the low-ratio groups. Solid lines represent cultures from feces, broken lines cultures from milk.

strong evidence that the groups, as we have arranged them, are legitimate. Howe does not publish his complete data, and while it is possible that his collection obtained from the digestive tract of man may have obtained some high-ratio cultures, it is evident that about half of his cultures were identical with our saccharose-raffinose-dulcitol fermenting group. It is probable that the remaining cultures may be classified with our non-saccharose fermenting group. The classification proposed by Jackson¹¹ and adopted by the American Public Health Association¹² includes under *Bacillus coli* only cultures of the colon type which ferment dulcitol, and divides these into *Bacillus coli communior*, which ferments saccharose, and *Bacilli coli communis*, which fails to ferment saccharose. There is no evidence, however, that these groups have any basis other than convenience in identification. We would suggest, therefore, that the low-ratio group, which as a whole has very definite characters and limitations, be designated as *Bacillus coli*, and that the varietal name *Bacillus coli communior* be applied to the saccharose-raffinose fermenting subgroup, and *Bacillus coli communis* to the group which fails to ferment these sugars. It should be noted that two individual cultures do not agree in all particulars with the classification outlined. One, jq, forms a pigment on agar and gives an exceptionally high acid formation in dextrose broth. Otherwise it does not differ from the typical cultures. The only other exception was ii, which belongs in the high-ratio group, and which was excluded from the correlation tables.

THE ABSENCE OF THE HIGH-RATIO GROUP

In the collection of gas-forming bacteria obtained from milk, 48 per cent. were sharply differentiated from the low-gas-ratio group by the high-gas ratio and certain fermentative reactions. We have seen, on the contrary, that only one culture of our collection from bovine feces could be placed with the high-ratio group. This may possibly be due to the fact that the high-ratio group occurred in such small numbers, in comparison with the low-ratio group, that only in one case were we able to pick off a sufficient number of colonies to secure a representative of the high-ratio group. It may also be possible that the methods of isolation were selective and tended to exclude one group. This is very improbable, as two temperatures and two media of quite different com-

11. *Jour. Am. Pub. Health Assn.*, 1911, 1, p. 930.

12. *Standard Methods for the Examination of Water and Sewage*, New York, 1912, p. 80.

position were used. Either group grows readily on gelatin, and all members of the high-ratio group which were tested grew well on the asparagin agar. A special effort was made to isolate from feces cultures which would give the characteristic high-ratio reaction. It was observed in the earlier results that adonite was fermented by 55 per cent. of the high-ratio group, but by only 15 per cent. of the low-ratio group. It would be expected, therefore, that if cultures were selected on the basis of the fermentation of adonite the chance of their belonging to the high-ratio group would be increased. Four samples of cow

TABLE 9
ANALYSES OF THE GAS PRODUCED FROM DEXTROSE BY BACTERIA OF COW FECES WHICH
FERMENT ADONITE

Organism	Total Gas in Cubic Centimeters	Percentage CO ₂	Percentage H ₂	Percentage Residual Gas	Ratio CO ₂ /H ₂
22ns	13.41	50.9	48.5	0.6	1.05
22nt	11.04	52.6	46.5	0.9	1.13
22nu	13.42	51.3	48.0	0.7	1.07
22nv	10.03	51.9	47.6	0.5	1.09
22nw	11.41	50.9	48.7	0.4	1.05
22nx	13.18	50.9	48.7	0.4	1.05
22ny	13.26	51.8	47.9	0.3	1.08
22nz	14.07	52.3	47.6	0.1	1.10

TABLE 10
GAS RECOVERED FROM 10 C.C. MEDIUM INOCULATED DIRECTLY WITH COW FECES AND
INCUBATED AT 30 C.

Sample	Total Gas	Percentage CO ₂	Percentage H ₂	Ratio CO ₂ /H ₂
5	2.01	51	47	1.07
5	5.48	52	47	1.09
6	2.19	52	45	1.16
8	4.02	52	46	1.13
8	4.10	52	46	1.13

feces were added to tubes of milk and of adonite broth, and incubated at 30 C. After twenty-four hours these tubes were plated on adonite-litmus agar and the acid colonies which developed were transferred to adonite-agar stabs. All of these stabs gave abundant gas.

Table 9 shows that none of the cultures so obtained could belong to the high-ratio group. A still more conclusive test was made by direct inoculation of five vacuum-fermentation bulbs with samples of cow feces. While it is probable that the growth of both types of the colon group would be suppressed by other bacteria, particularly by the strep-

tococci, we would expect to find, if the high-rate group was present, some evidence of an increasing proportion of carbon dioxide.

The following analyses of the gas produced in 10 c.c. of Broth 1, while they do show that a suppression of the production has taken place, do not indicate the presence of any high-ratio gas producers in the cow feces.

The failure of these special methods to give any evidence of the presence of high-ratio bacteria makes it reasonably safe to conclude that members of this group occur only rarely in the bovine intestinal tract.

CONCLUSIONS

These results give additional evidence of the constancy of bacterial reactions when they are determined under uniform conditions and by exact methods. The $\text{CO}_2 : \text{H}_2$ ratio, which has been generally discarded as valueless on account of its great variation, varied from 0.98 to 1.20 in determinations on 149 cultures. This is, no doubt, due to the fact that we have under consideration a closely related natural family which, when it ferments carbohydrates, is able to do so by one set of reactions only. These reactions are repeated by each individual of the family with mathematical accuracy, and variations from the theoretical end-products are due to lack of refinements in our methods, or to extraneous factors which use up some of the end products. The same conditions which have produced a family bringing about these particular reactions have also impressed on the group the ability to utilize certain carbohydrates and alcohols as sources of food or energy. It is evident that through some peculiarities in the protoplasmic molecule, the ability to ferment one substance carried with it the ability to utilize certain other substances also, and in some cases the inability to ferment others. However, the ability or inability to ferment certain substances is not so basic a function as the power of producing from these substances a definite chemical reaction with constant end-products. Consequently, we have more variation in the former function, a variation which produces subfamilies giving in common the same kind and proportion of end-products, but differing in the materials from which they are able to obtain them. The minor variations in the volume of gas produced under definite conditions are probably due to the variation exhibited by different cultures in ability to withstand the acid produced by their own activities. Consequently the evolution of gas ceases earlier in some cultures than in others. There is, however, a close correlation between

the gas volume and $\text{CO}_2:\text{H}_2$ ratio, and the volume is evidently a distinctive characteristic of some families. These families are doubtless produced under the long-continued influence of uniform conditions, and when we begin to select our cultures from a definite habitat it becomes easy to establish natural groups with remarkably constant and typical characters. Thus these results show that the bovine intestine is the habitat of two distinct but closely related varieties of the colon group, just as an earlier paper¹³ from this laboratory has shown that a distinct variety of the streptococci can be obtained from the same source.

The negative results have an interest second only to the establishment of the limitations of *Bacillus coli* group as it occurs in bovine feces. Of 125 cultures isolated from milk and answering all of the usual presumptive tests for *Bacillus coli*, almost one-half possessed characters which differentiated them sharply from the type found almost exclusively in bovine feces. Only one of 150 cultures from bovine feces gave reactions which identified it with the high-ratio group, which made up nearly 50 per cent. of the milk cultures. These results are surprising as there is no apparent reason why the high-ratio group, which, in our opinion, is identical with the so-called aërogenes-lactici acidi group, should not grow in the intestinal tract with its relative, *Bacillus coli*. Nevertheless, our efforts to isolate more than a single culture have so far failed, and the obvious conclusion, assuming the correctness of our results, is that the habitat of the high-ratio group must be sought elsewhere. An attempt will be made in this laboratory to obtain a definite answer to this question.

SUMMARY

Previous work on a collection of the colon type from milk demonstrated that the gas ratio and volume are constant under uniform conditions; that, on the basis of the gas ratio and volume, the cultures may be divided into two distinct groups; and that the correlation of the fermentative ability with the gas ratio makes this distinction sharply defined.

This paper records the results of a similar study on 150 cultures isolated from bovine feces. None of these cultures liquefied gelatin and all but one formed indol from tryptophan. By the use of a simple medium and exact methods of analysis, it was found that in 149 cul-

tures the $\text{CO}_2:\text{H}_2$ ratio varied only from 0.98 to 1.20. One culture only gave a ratio identifying it with the high-ratio group, which made up 48 per cent. of the milk series.

The 149 low-ratio (0.98-1.20) cultures were readily divided into two groups, one of which fermented dextrose, saccharose, lactose, raffinose, mannite, glycerin and dulcite, but almost invariably failed to ferment starch, inulin and adonite, while the second group fermented adonite and dulcite and failed to ferment saccharose, raffinose, starch and inulin.

These groups agree almost perfectly with two groups which may be formed from the low ratio cultures isolated from milk. Special methods failed to give evidence, with the exception of the single culture mentioned, of the presence in bovine feces of the high-ratio group, which made up about one-half of the milk collection.

THE TOXINS OF INTESTINAL OBSTRUCTION *

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During the past two years a very considerable number of valuable observations have been made on the nature of the factors which underlie the fatal termination so frequently encountered in cases of obstruction of the intestinal tract. Previous to 1911, although the fact that occlusion of the gut lumen is followed, within a comparatively short time, by a characteristic chain of symptoms—especially those commonly associated with shock—was generally recognized, relatively little experimental work had been done which would throw light on the causes of death in this affection. Within the last two years, however, several groups of investigators have approached the question experimentally from different points of view, and so at the present time this subject appears to be rapidly approaching a solution.

It is the author's intention to record a certain number of experiments which he believes to have a direct bearing on this subject, rather than to enter into a detailed consideration of the various data obtained by different writers on the subject.

Of the views relative to the mode of death in cases of simple acute intestinal obstruction that which assumes a bacteriemia has been pretty generally disproven and is now abandoned. The remaining opinions are those of Hartwell and Hoguet¹ "that the enormous loss of water due to the vomiting is the essential factor," and that of Whipple, Stone and Burnheim who claim that death in cases of intestinal obstruction is due to the absorption from the lumen of the gut of a toxic substance, as yet not positively identified, which is potent to lead to the death of normal animals if injected intravenously or intraperitoneally.

Briefly, Hartwell and Hoguet in their experiments took pains to so occlude the gut that the vascularization of the latter was not interfered with and under these circumstances the death of the animal was evidently due, chiefly at least, to starvation and, more particularly, to withdrawal of fluids from the tissues. Death, moreover, takes

* Received for publication February 6, 1914.

1. *Am. Jour. Med. Sc.*, 1912, 143, p. 357; *Jour. Am. Med. Assn.*, 1912, 59, p. 82.

place under such circumstances at the end of from three to ten days, while animals which receive large quantities of salt solution subcutaneously live for three weeks or more. As Vincent² has pointed out, such experiments resemble more the chronic forms of intestinal obstruction seen clinically, such as the form due to extra- and intra-intestinal tumors.

In the work of Whipple and his associates³ isolated loops of intestine were employed. With closed, washed duodenal loops, dogs die in from twenty-four to forty-eight hours with symptoms of most profound intoxication, as evidenced by a lowering of temperature and marked drop in blood pressure, and at autopsy they present evidence of extreme splanchnic congestion. During life such dogs suffer from a bloody diarrhea more particularly if a gastro-enterostomy is performed, in which case there is also marked vomiting.

If the isolated duodenal loop is drained the evidence of intoxication is much mitigated and the dogs live for weeks or even months.

The contents of the isolated loops are usually of the consistency of pea-soup, pinkish or anchovy-sauce like in color, and are of a characteristic heavy penetrating odor. After this material is removed, heated to 60° C. to destroy bacterial life, filtered, and the filtrate introduced into a normal animal, it causes death in from three to twelve hours, or immediately, if a sufficiently large dose is employed. The animal manifests symptoms closely allied to those which occur when the closed-loop experiment is performed. There is a similar evidence of shock and diarrhea, the latter sometimes bloody. Postmortem there is the same evidence of splanchnic paralysis.

Murphy and Vincent⁴ are of the opinion that the difference in the symptoms following occlusion without vascular disturbance, and those experiments in which the vessels, more particularly the veins, are interfered with, is due to alteration in the nutrition of the mucosa permitting absorption of intestinal contents to take place.

Two points, therefore, appear to have been established: (1) that under certain circumstances of occlusion of the gastro-intestinal tract, reflex or toxic vomiting may be so marked as to lead to the death of the affected animal as the result of the consequent withdrawal of water from the tissues, and (2) that there is also produced in closed loops of intestine a toxic substance which rapidly leads to the death

2. *Jour. Am. Med. Assn.*, 1912, 59, p. 86.

3. *Johns Hopkins Hosp. Bull.*, 1912, 23, p. 159; *Jour. Exper. Med.*, 1913, 17, pp. 286, 307.

4. *Jour. Am. Med. Assn.*, 1912, 59, p. 86.

of the animal, and which acts chiefly through inducing extreme splanchnic congestion.

The author's experiments, a certain number of which are given later, prove that it is possible to induce the production in the mucosa and within the intestinal lumen, of a similar poisonous body although there is no mechanical obstruction to the gut lumen.

These experiments are the outcome of an observation of the similarity in gross appearance of the intestinal contents, and of the manner of death in dogs which had been operated on by Dr. E. M. von Eberts and Dr. W. H. P. Hill in an attempt to partially occlude the lumen of the portal vein by means of a fascial band. Although these experiments were undertaken with an altogether different end in view, it was noted that in those animals in which the band was applied so tightly as more or less completely to obliterate the vein, death occurred within fifteen to thirty-six hours, and that at autopsy the intestines contained a fluid similar to that found in cases of acute intestinal obstruction. This material was tested after the manner of Whipple's experiments and was found to possess similar toxic properties. Since, in these cases, the development of the poisonous material depended exclusively on vascular disturbance of the gut wall a number of animals were operated on and the portal veins or its branches ligated. In all of these operated animals the same type of fluid, which possessed the same toxic properties as the fluids found in closed duodenal loops, was discovered.

Dogs in which the portal vein, or the branches thereof draining the upper half of the intestinal tract, was ligated, died regularly within fifteen to eighteen hours. The animals never completely recovered from the stupor of anesthesia and were frequently found dead in exactly the same position in the cage as when returned from the operating room. The intestines, pancreas, and kidneys were found deeply cyanosed and the heart blood unduly fluid. The lumen of the intestine contained fluid blood-stained material, as a rule of a more watery consistency and deeper purple color than that found in cases of gut occlusion. The intestinal contents were collected from such animals and the cyanosed mucosa scraped off and placed in saline solution under toluol for extraction. The result obtained by employing this extract for intravenous inoculation into normal dogs is given in the following protocols.

Experiment 5 T.—Collie bitch, weight 40 lbs. Injection made into left femoral vein of 25 c.c. filtered fluid prepared from intestinal scrapings and blood-stained contents from Dog 4 T. Scrapings and contents of mucosa were preserved for 5 days before use in the ice chest under toluol.

During incision of thigh and isolation of vein, the dog was under light ether anesthesia but was permitted to "come out" of the anesthetic before the injection was made. Within 15 seconds before the completion of the injection, the dog became restless but almost immediately manifested evidence of most profound shock. The pulse at first became accelerated so that the beats could not be counted, but within 2 minutes the pulse became slower, and there was a marked drop in blood-pressure, as evidenced by the absence of pulse beats in the femoral artery. Death occurred in 2 minutes from the time of injection.

Immediate autopsy showed extreme venous congestion of liver, pancreas, intestinal tract, more particularly the lower part of the stomach, the duodenum and upper portion of the ileum, and the kidneys.

Experiment 12 T.—Terrier dog, 23.5 lbs. Injection of toxic material extracted from intestinal mucosa of Autopsy 152, Experiment 9 T. Material prepared by extraction of washed intestinal mucosa, in an equal weight of physiologic saline for 6 days in an ice chest under toluol with subsequent filtration. Moderate to slight autolysis of the mucosa had occurred during this time. Sphygmographic tracings were made during the early part of this experiment.

The dog received 20 c.c. of the solution in the left femoral vein, and within 90 seconds a marked drop in blood-pressure (40 mm.) occurred, accompanied by a slight increase in the pulse rate and diminution in the excursion of the pulse wave. Within 8 minutes the blood pressure had returned to normal and the dog appeared normal. He was then given a second injection of 22 c.c. which was followed by a repetition of the previous symptoms, culminating in death 1 minute after completion of the injection.

Autopsy was performed at once and revealed the characteristic evidence of splanchnic congestion.

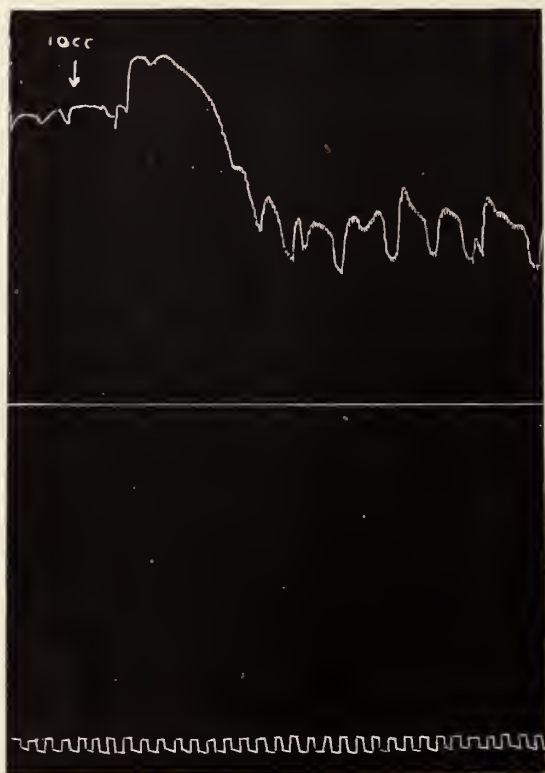
Experiment 8 T.—Terrier bitch, weight 18 lbs. Injection of 20 c.c. of filtered material prepared by extracting, in an equal weight of salt solution, mucosa from a dog, in which the portal vein had been tied. Dog showed characteristic evidence of intoxication, was very restless, and cried out. Recovery was apparently complete in 10 minutes. Eighteen hours later the animal appeared fairly well but soon showed a blood-stained diarrhea and collapsed on the floor 22 hours after injection.

Autopsy showed heart in diastole filled with fluid blood. There was moderate congestion of the liver, pancreas and upper intestinal tract. The gut lumen contained a considerable quantity of blood-stained fluid.

Experiment 15 T.—Terrier bitch, weight 16 lbs. Injection of toxic material recovered from Experiment 14 T. This material was prepared as follows: 75 c.c. of the typical material from the gut lumen was added to 25 gm. of mucosa, plus 100 c.c. of physiologic saline. This was placed in the ice chest under toluol for 48 hours, centrifuged and filtered. The experiment was carried out while the animal was attached to the blood-pressure recording apparatus. In all, the animal received 45 c.c. of the diluted material in four doses—15 c.c., 15 c.c., 10 c.c., and 5 c.c. The characteristic effect of the injection is well shown in the accompanying tracing. It will be noticed that there is constantly produced a moderate initial rise in blood-pressure which precedes the marked drop noted clinically.

It is further noted that there appears to be but little cumulative effect of the toxin, at least in so far as the primary effect is concerned, nor is there any tolerance induced by repeated doses at short intervals. As the animal was not killed by doses of this size it was chloroformed.

Autopsy revealed the most extreme grade of cyanosis and congestion of the splanchnic area, simulating the appearance, in so far as the intestines and pancreas are concerned, when the portal vein is ligated. The intensity of the congestion is shown by the fact that the liver, which was removed after clamping



Copy of sphygmographic tracing, Experiment 15T, showing fall in blood-pressure following initial rise after the injection of 10 c.c. of extract.

the vessels, decreased in weight from 520 to 493 gm., when the blood was allowed to flow out, by application of gentle pressure after removing the clamps. There had thus been a gain in weight, due chiefly if not exclusively, to increased blood content, of 26 per cent.

Whipple states that he has been unable to isolate a similar toxic product from normal intestinal mucosa. The author's experiments in this direction are very limited, but Experiment 16 T indicates that it

is possible for a similar toxic body to be recovered from autolysis of normal mucosa.

Experiment 16 T.—Terrier dog, weight 10 lbs. 4 oz. Injection intravenously of 25 c.c. of an extract of normal dog's intestinal mucosa membrane. This extract was prepared by adding washed mucosa to an equal weight of salt solution and storing under toluol in the ice chest for 3 days. There was no odor of putrefaction nor did gross evidence of disintegration of the tissues occur in this length of time. The dog received 25 c.c. of the extract intravenously. Within 2 minutes the animal cried out and the pulse became rapid and weak. Then ensued gradual recovery after 2 or 3 minutes and at the end of 10 minutes the dog appeared normal. The following morning he appeared to be well up to 10 a. m., but was found dead at noon.

Autopsy revealed moderate venous congestion of the intestines, more particularly the stomach and duodenum. The lumen of the gut contained bloody, pasty, and fluid material.

There remain many features with regard to the nature and mode of production of the toxic substance which require further study and elucidation, including investigation such as that carried out by Whipple and his associates into the development of immunity against the toxin. The foregoing experiments are recorded simply because they appear to indicate certain factors which determine the production of the substance and in this way suggest the nature of the toxic material and its source.

CONCLUSIONS

Thus it has been proven that vascular disturbances, namely, venous stasis, are equally as potent as occlusion of the lumen in the development of a poisonous substance in the intestinal mucosa, and that the substance produced in this manner is likewise a splanchnic paralyzer.

Since the mucosa itself appears to contain a greater content of the toxic principle than does the content of the lumen, it would appear that the substance responsible for the intoxicating symptoms is the result of tissue autolysis rather than a bacterial product.

It remains to be proven whether the death of the individual in cases of portal obstruction is due to the absence of the liver activity or to the development and absorption of the intestinal toxin, or of a combination of these two factors, and the foregoing experiments indicate that the effect on the nutrition of the intestine may be a sufficient cause for the untoward manifestations.

THE RELATIONSHIP OF SEPTIC SORE THROAT TO INFECTED MILK *

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The epidemic of streptococcus sore throat which visited Chicago during the winters of 1911 and 1912 was definitely traced to one dairy.¹ This dairy had its largest collecting and pasteurizing plant in the town of Batavia, and the milk was delivered to this plant from about eighty-five dairy farms in the adjacent country. On a number of these farms were found not only cases of mastitis in the cows, but cases of sore throat among the milkers, which were of the same type as the epidemic cases in Chicago using milk from this dairy.

In the published report of the Chicago epidemic, reference was made to the coincident outbreak in Batavia, but the investigation of the latter had not been completed. This final report reveals certain unusual conditions prevailing in Batavia, that seemed to have greatly increased the spread of the disease in that town.

The small epidemic in Oak Park herein described, occurred during the same winter as those in Chicago and Batavia, but had an independent origin. The report is of interest because the investigation was started from the infected cow and followed to the milk consumers, instead of being started in the usual way from the consumer and traced to the cow.²

THE RELATION OF THE EPIDEMIC IN BATAVIA TO THE MILK-SUPPLY

The milk brought in from the country to the pasteurizing plant of Dairy X at Batavia, was sent to the town of Batavia as well as to Chicago. A study of the pasteurizing records convinced us that during the months under consideration (December, 1911, to March, 1912), the apparatus was inefficient on many days. An extensive canvas in four different sections of Chicago showed that the sore throat morbidity among users of milk from this dairy was from seven to fourteen times as great as among users of milk from other dairies. It seemed

*Received for publication Feb. 9, 1914.

1. *Jour. Am. Med. Assn.*, 1912, 58, p. 1848.

2. *Ibid.*, p. 1852.

important, therefore, to determine whether users of this milk in Batavia also exhibited a relatively high morbidity of sore throat.

An investigation was made of the prevalence of sore throat and of the milk-supply in 494 households. It was found there were 218 cases of sore throat in 140 households using milk from Dairy X, or a ratio of 156 cases to 100 households supplied by this milk. This was even higher than the ratio obtained in Chicago, viz., 113 cases of sore throat to 100 households supplied.

But at this point the Batavia epidemic offered a decided contrast to the one in Chicago. In Chicago the customers of other dairies had a low morbidity, whereas in Batavia, sore throat was prevalent among the customers of the 11 other dairies, altho in no case did it approach the high morbidity of Dairy X. The ratios in the 11 dairy routes varied from 3 cases of sore throat to 100 households to 64 cases to 100 households.

This unusual prevalence of sore throat among the consumers of milk of other dairies was not explained by examination of the cows and milkers.

Mastitis was reported on four farms. Individual cases of sore throat were found on two farms. On one farm only, Dairy G, was the combination of sore throat and mastitis revealed and this farm was one of the smallest, supplying only fourteen households. The great majority of cases of sore throat used milk from dairies presenting no suspicious conditions on the farms.

A discovery by the dairy inspector of the Health Department threw a new light on the situation. He frequently noted at the dairy farms other dairies' bottles which were taken in exchange at customers' houses. These bottles were washed merely in cold water. Since none of these eleven dairies pasteurized their milk, an infected bottle would thus be capable of contaminating pure milk.

Chart 1 shows that Dairies A-G, inclusive, delivered milk in bottles, and on every one of these farms excepting Dairy G, bottles belonging to Dairy X were found.

Dairies H, K, L and M delivered milk in cans or pails and consequently their milk was not exposed to bottle contamination. The ratio of cases of sore throat among the can users was 17 cases to 100 households, compared with 37 to 100 among the bottle users, and 156 to 100 among the consumers of Dairy X milk. To express the matter differently, over 77 per cent. of all cases of sore throat among custom-

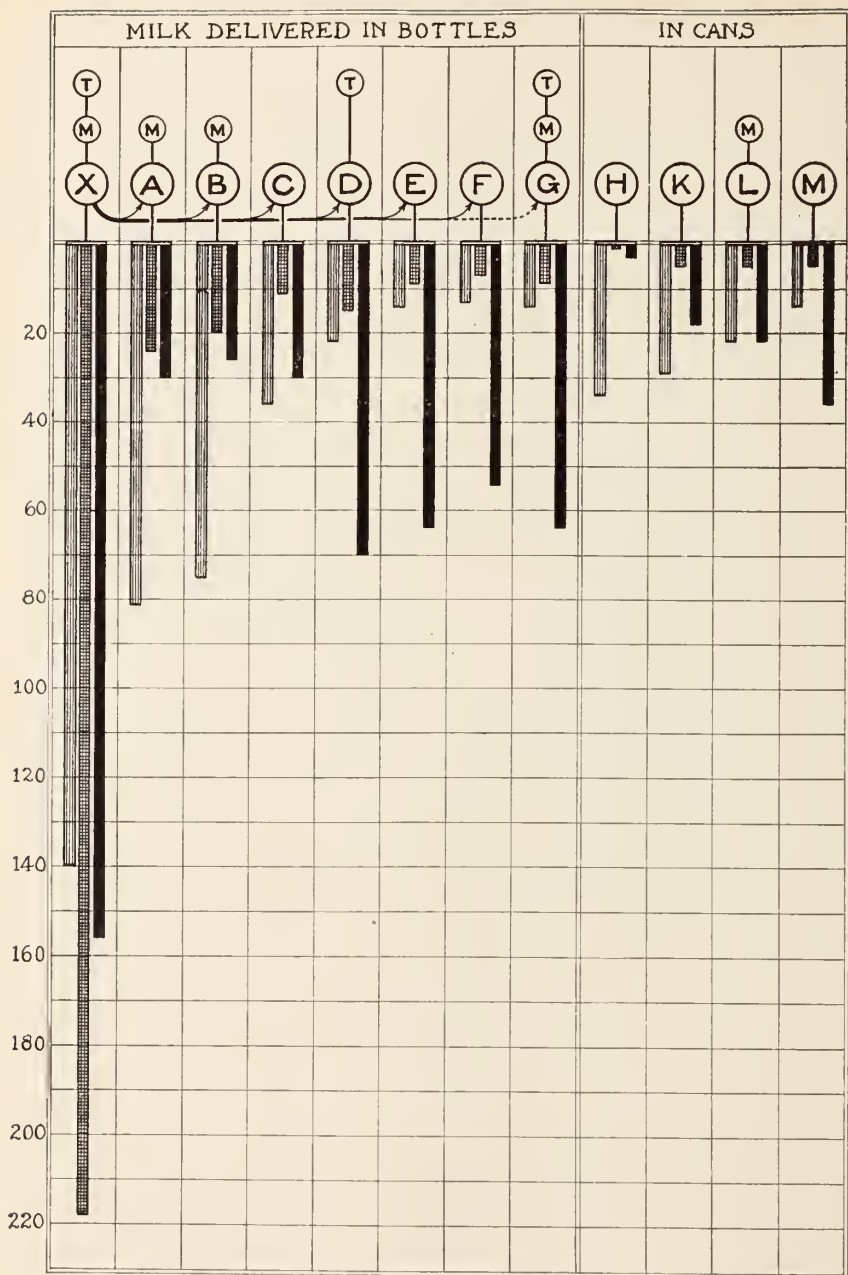


Chart 1.—Illustrating the proportion of sore throat cases credited to 12 dairies of Batavia. The letters T and M in small circles = sore throat and mastitis respectively on the farm; the column of vertical lines = the number of households supplied; the barred column = the number of cases of sore throat; the black column = the number of cases per hundred households. Dairies A—F are shown by arrows to be exposed to contamination by exchange of bottles with Dairy X, which is known to be infected.

ers of the eleven dairies A-M were exposed to the contamination of bottles from Dairy X.

This evidence is submitted not as conclusive, but as highly indicative of a wide-spread milk contamination though a free exchange of bottles with a dairy whose milk was infected.

THE EPIDEMIC IN OAK PARK TRACED FROM THE FARM TO THE CONSUMERS

As stated before, we have looked on the combination of mastitis in cows and sore throat in the milkers on the same farm as strong circumstantial evidence of infected milk. We thereupon searched for a farm, where such a combination of conditions was present, and found it.

The farm was stocked with fifteen cows, three of which were affected with mastitis during January and February, 1912. At the time of our visit one cow still had a caked bag and the milk was being saved for consumption. In the household were twelve persons, including employees, nearly all of whom used milk or cream. Within the above mentioned period nine of these individuals were ill with sore throat.

The milk was sold to Dairy A (Chart 2) in Oak Park. Two other farms supplied milk to Dairy A, but on neither of these farms (Farms 2 and 3) were any cases of sore throat or mastitis found.

Dairy A delivered the mixed milk from these three farms in bottles without pasteurizing. A careful canvas of the 140 customers of Dairy A in Oak Park showed 40 cases of sore throat, or 28 cases to 100 households supplied.

To establish a basis of comparison a list of customers was obtained in the same neighborhood from Dairy B, which employs the holding method of pasteurization. In a total of 84 households supplied by Dairy B, five cases of sore throat were found, or less than six cases per 100 households.

There were four and one-half times as many cases of sort throat, proportionately, among the customers of Dairy A as among those of Dairy B.

We believe that the excessive morbidity among the users of Dairy A milk was probably due to the consumption of milk which was contaminated on Farm 1.

Is it not reasonable to suppose that similar small epidemics are of frequent occurrence?

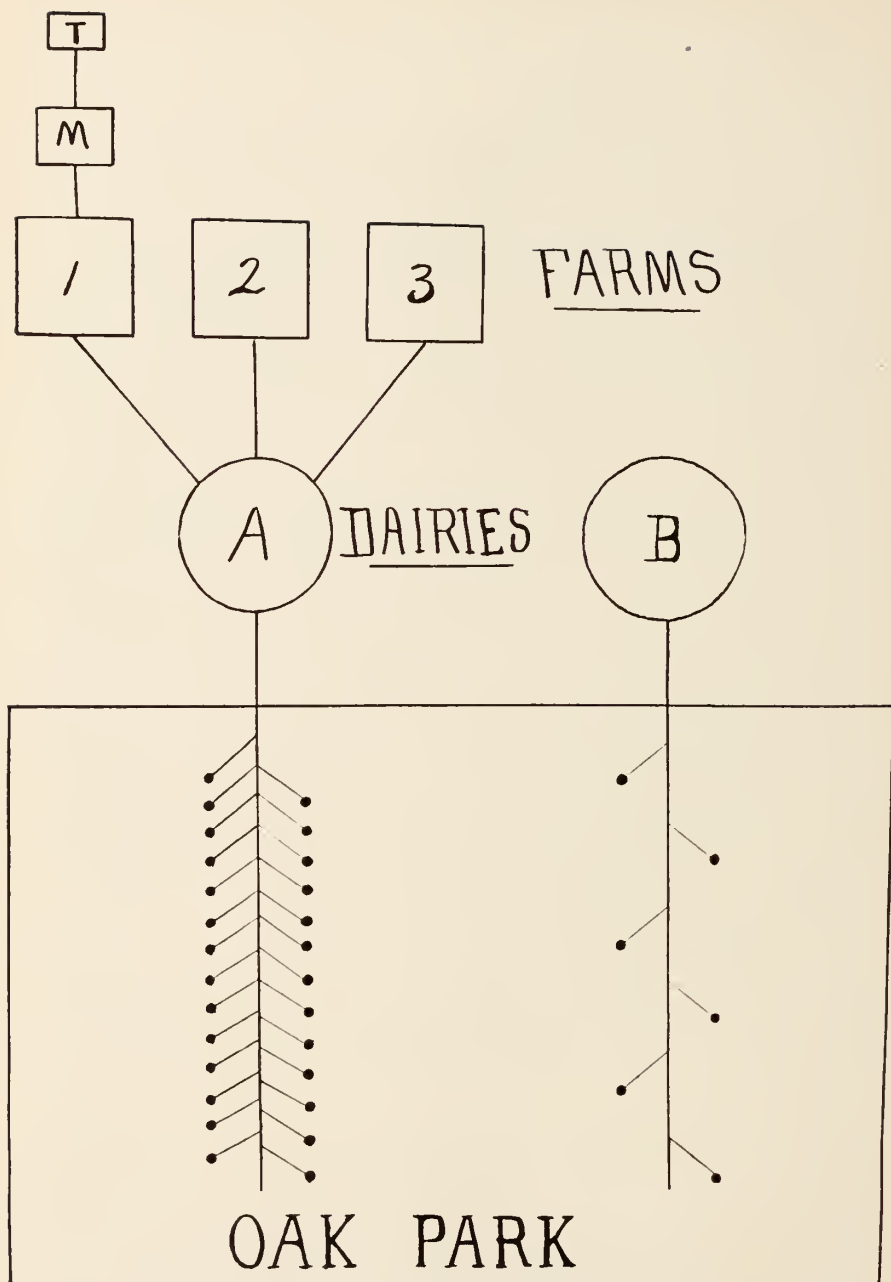


Chart 2.—Tracing the milk from a suspected farm, Farm 1, where mastitis^o (*M*) is prevalent in the cows and sore throat (*T*) in the milkers to a dairy company in Oak Park (*A*), which does not pasteurize. Compare the number of sore throat cases per hundred households (27) occurring in this route to the small number (6) occurring in another dairy route in the same neighborhood.

EXPERIMENTAL BOVINE MASTITIS PRODUCED WITH HEMOLYTIC STREPTOCOCCI OF HUMAN ORIGIN *

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The Chicago epidemic of septic sore throat reported by Capps and Miller¹ was traced to a contaminated milk-supply at Batavia, Ill. It was found that on a number of farms supplying milk to Dairy X, sore throat occurred in the milkers, and mastitis appeared in the cows; in some instances the sore throat appeared first, and in other instances the mastitis preceded the throat infections. These facts bring up the question whether or not it is possible for milkers suffering with sore throat to infect the teats or udders of cows by contaminated hands or otherwise during the process of milking. The question of the susceptibility of the cow to human streptococci is also involved. In an attempt to clear up these points some experiments were designed to test the pathogenicity of human streptococci for cows, and to determine possible avenues of udder infection through the teats.

For this purpose a healthy milch cow was obtained, giving at the time about 16 quarts of milk in twenty-four hours. Examination of the udder revealed no lesions, and examination of the milk from each quarter after proper cleansing of the teats showed no hemolytic streptococci on blood agar plates. Leukocytes in the milk from the quarters numbered from 10,000 to 30,000.²

Experiment 1.—On three successive days the uninjured surfaces of the teats of the cow were smeared with milk cultures of typical hemolytic streptococci, recently isolated from cases of streptococcus tonsillitis. Two different strains were used. The result was entirely negative. Later, the teats were similarly smeared with a culture of streptococcus isolated during the milk epidemic from a fatal case of streptococcus infection. The result was also negative. A further experiment was made by smearing on the uninjured surface of the teat some grayish exudate teeming with streptococci which had just been removed from the tonsil of a severe case of tonsillitis. Examinations were made of both the first and last milk each day for a period of a week following these applications, but there was no evidence of infection. Streptococci were not found, and no increase in leukocytes appeared in any of the udder quarters.

* Received for publication February 9, 1914.

1. *Jour. Am. Med. Assn.*, 1912, 58, p. 1848.

2. The blood agar plate method was used in all the bacteriological examinations reported in this paper and the Doane-Buckley method was employed for counting leukocytes.

Experiment 2.—A slight abrasion of the skin, sufficient to cause slight bleeding, was made at the end of one of the teats near the meatus. A culture of a hemolytic streptococcus was then directly applied. The wound became infected locally and there was slight swelling, redness, and evident tenderness. In the first milk some hemolytic streptococci appeared almost immediately, but they were practically absent from the last milk. After several days the streptococci appeared in large numbers and about the tenth day the leukocytes were markedly increased. For instance, in the first milk on the day following the infection the hemolytic streptococci in the milk from the involved quarter numbered 1,500 per c.c.; on the second day 5,000, and on the third day 10,000 per c.c. They continued at about this number for some time and then became less. At the end of four weeks they were still present in numbers from 100 to 500 per c.c. Milk from the control quarter gave no streptococci. The leukocytes from the infected quarter, most of which were typical polynuclear cells, on the ninth day after inoculation numbered 250,000 per c.c., and on the twelfth day 1,408,000 per c.c. For a time they remained about the same or slowly decreased, but at the end of one month they still numbered 100,000 per c.c. It is to be noted that at no time was the udder caked or swollen to any extent and milk obtained from this quarter was not "gargety" or stringy. From this experiment it appears that it is possible for the udder to be infected with human streptococci from an abrasion on the teat apparently by a direct infection ascending the canal.

Experiment 3.—In this experiment two twenty-four hour milk cultures of a hemolytic streptococcus, recently isolated from a typical case of ordinary follicular tonsillitis, were introduced into the udder by means of a catheter inserted to a distance of about 8 cm. above the meatus. The amount of culture injected was 8 c.c. On the following day there was evidence of inflammation manifested by slight but distinct swelling of the quarter, some tenderness, and the presence of "gargety" milk. The quarter was not caked or hard. Enormous numbers of leukocytes were present and large numbers of typical hemolytic streptococci, especially in the first milk. They were present but were less numerous in the last milk. The streptococci continued to be present in the milk for several weeks and the marked increase in the number of leukocytes also continued for about the same length of time. Table 1 shows the number of streptococci and leukocytes per cubic centimeter from day to day in the milk from the infected quarter.

During the course of the experiments the cow remained in good physical condition. No appreciable increase of temperature was noted and there was no emaciation. The milk flow from the involved quarters was distinctly decreased during the infection but it later increased to the normal amount. The entire milk flow decreased during the course of the experiment rather more perhaps than one would expect under normal conditions. Lymph glands about the infected quarters were enlarged for a few days during the acute stage of the infection. There was no evidence that the infection spread from one quarter to another during the experiments. After about six weeks or two months, the teats and all the quarters of the udder became normal.

A point of interest in connection with these experiments is the possible effect that the milk might have on the properties of the streptococcus in the udder. This is important because it was noted that the streptococci isolated from many of the cases during the epidemic of sore throat had some rather characteristic properties which

differentiated it from ordinary hemolytic streptococci. These were, briefly, its relative narrow zone of hemolysis, its moist mucoid growth, and the presence of a capsule. Careful observations were made of the streptococci from day to day as they were isolated from the milk on the blood agar plates. No change of properties whatever was noted. The streptococci, which were typical hemolytic streptococci when first isolated from the human throat, revealed no modification as to hemolysis, capsules, virulence, etc., even after growing in the udder for over four weeks. They still readily produced arthritis in rabbits.

TABLE 1.

THE NUMBER OF STREPTOCOCCI AND LEUKOCYTES IN THE MILK AFTER INTRODUCTION OF STREPTOCOCCI IN THE UDDER (EXPERIMENT 3).

No. of Days After Injection of Streptococci	No. of Streptococci per 0.1 c.c.	No. of Leukocytes per c.c.
0	0	12,000
1	200	9,840,000
2	500	8,500,000
3	3,000	5,200,000
4	1,200	1,320,000
5	500
6	150	624,000
7	300
8	1,500	200,000
9	240	275,000
10	175	936,000
11	500	472,000
12	200	360,000
13	100	320,000
14	400	1,472,000
15	14	512,000
16	20	136,000
17	14	448,000
18	—	—
19	—	—
20	30	944,000
21	—	—
22	11	780,000
23	—	—
24	2	260,000
25	—	—
26	—	—
27	—	—
28	0	190,000

Milk which came from the infected udder and contained many streptococci was fed in large amounts to rabbits, guinea-pigs and a monkey. After a period of from two to three weeks several guinea-pigs developed multiple arthritic lesions. In the affected joints there was swelling associated with hemorrhages. The rabbits and the monkey did not develop arthritis or other lesions. This point is now being more carefully investigated and feeding experiments with milk and with streptococci are being carried on to find out, if possible,

what factors in the feeding experiments were responsible for the lesions in the guinea-pigs.

After carefully cleansing the meatus and removing the first milk it was possible to obtain a pure growth of streptococci from the infected udder. This was used as a basis to carry out some experiments to show the effect of temperature on the growth of these organisms in milk. The observations were made on the streptococci not only when growing pure in milk obtained directly from the udder, but also when growing in milk along with the ordinary contaminating bacteria.

TABLE 2.

THE EFFECT OF REFRIGERATOR, ROOM AND INCUBATOR TEMPERATURES ON THE GROWTH OF PURE STREPTOCOCCI IN MILK

Temperature	At Once	½ Hr.	1 Hr.	3 Hrs.	6 Hrs.	20 Hrs.	48 Hrs.
Refrigerator	1,100	1,300	1,300	1,300	1,400	1,300	1,000
Room (20-22° C.)	1,200	1,200	1,300	1,300	1,500	7,000	—
Incubator (37° C.)	1,200	1,200	1,300	10,000	20,000	—	—

Table 2 shows the results obtained by permitting the milk containing pure streptococci to remain at refrigerator, room and incubator temperatures, plates being made at intervals in order to determine the number of organisms from time to time.

An examination of the table shows that the number of streptococci at refrigerator temperature remains practically the same for forty-eight hours. At room temperature (20 C.) there is some increase after six hours, and a decided increase at the end of twenty and forty-eight hours. In the incubator the multiplication of colonies is rapid, the number having increased tenfold after three hours. This result is about what one would expect with a pathogenic organism, such as the streptococcus.

TABLE 3.

THE EFFECT OF THE PRESENCE OF CONTAMINATING BACTERIA ON THE GROWTH OF STREPTOCOCCI

Temperature	At Once	½ Hr.	1 Hr.	3 Hrs.	6 Hrs.	22 Hrs.	48 Hrs.
Refrigerator							
Streptococci	39	64	45	55	67	72	80
Other bacteria	16	22	27	24	34	23	27
Room (20-22° C.)							
Streptococci	92	40	79	97	66	260	250
Other bacteria	48	100	120	116	84	1,960	10,000+
Incubator (37° C.)							
Streptococci	46	78	67	106	111	3,200	120
Other bacteria	9	31	27	24	16	232	10,000+

Table 3 shows the effect of the presence of other bacteria on the growth of streptococci in mastitis milk. These bacteria are the ordinary contaminating bacteria found in milk when no special precautions are taken in the process of milking. They include the streptococcus lacticus, colon bacilli, etc., organisms which ordinarily are not pathogenic.

At refrigerator temperature, as one would expect, no appreciable increase of bacteria appears; at room temperature (20 to 22 C.) there is only a slight increase in the number of streptococci but a very great increase in the number of other germs. At incubator temperature the number of streptococci increase considerably in twenty-two hours, but at the end of forty-eight hours a marked diminution occurs. The other germs, however, show a marked increase at the end of forty-eight hours. Several similar experiments were made; there was noted some variation in the number of colonies both of streptococci and of other organisms at room temperature and at incubator temperature after twenty-four and forty-eight hours. This was due to the fact that the contaminating bacteria vary in number and kind and they may grow better at room temperature than at incubator temperature. However, in general, at the end of twenty-four and forty-eight hours there was a tendency for the contaminating bacteria to overgrow or inhibit the growth of the streptococci, as is readily seen by comparing Tables 1 and 2. These data do not indicate that a brief exposure of milk containing streptococci to a favorable temperature, as might occur for example in the process of pasteurization, causes any appreciable increase in the number of bacteria.

In connection with these experiments, it should be stated that a number of tests showed that the streptococci in milk as it comes from the infected udder are readily killed by an exposure of twenty to thirty minutes at 60 C. (140 F.).

Of interest in connection with experimental mastitis is the onset, also the duration of the infection which, as we have seen, exists for several weeks at least. There is every reason to believe that the onset and duration of streptococcal mastitis as it occurs under natural conditions correspond to the onset and duration in experimental mastitis. These facts may be brought into relation with the onset and duration of the milk epidemics in which the outbreak is usually explosive in character and the duration usually continues for a number of weeks. The Chicago epidemic began suddenly about December 21, reached

its maximum December 25, and then with the exception of a sudden rise on January 1, more or less gradually subsided, lasting about five or six weeks. The courses of the Boston and Baltimore epidemics corresponded in a general way to that of the Chicago epidemic, and the same may be said of the milk epidemics reported in England. It is seen, that there is a rather striking agreement between the onset and the duration of the experimental mastitis and the onset and duration of the milk-borne epidemics of septic sore throat.

SUMMARY

Hemolytic streptococci of human origin may cause mastitis, lasting for several weeks in cows. This time roughly corresponds to the duration of milk-borne epidemics.

The streptococci may gain entrance through an abraded or injured surface of the teat. It appears possible therefore for mastitis in cows to be produced by an infection from the milker whose hands are contaminated perhaps from a sore throat.

Mastitis results promptly when the cocci are injected directly into the udder by means of a catheter.

The mastitis may exist without physical evidence. A caked bag may not occur, though pus and streptococci in large numbers are being secreted in the milk.

Stringy, ropy, or gargety milk may or may not occur during the course of the mastitis.

In order to detect such infections, it might be necessary to examine milk from each quarter for bacteria, and for pus. This may explain the failure to detect the source of the streptococci in some of the epidemics of sore throat.

No change was noted in the cultural or pathogenic properties of the streptococci after growing in the udder of a cow for four weeks.

The presence of various contaminating bacteria tend to inhibit the growth of hemolytic streptococci in milk.

AN INVESTIGATION INTO THE FERMENTATIVE ACTIVITIES OF THE ACIDURIC BACTERIA *

ALFRED H. RAHE

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The wide use of the bacillus bulgaricus, both topically and by mouth, to control intestinal putrefaction has within the last few years given rise to a number of investigations aiming to give a definite classification to this group of lactic acid bacilli. It would seem that this could be satisfactorily accomplished by a systematic examination of their prominent cultural characteristic, namely, the fermentation of carbohydrates. That this means of separation has not been successfully used heretofore is probably due to the assumption that these bacteria will not grow on the usual laboratory media, though mention is made by the earlier workers of growth in glucose and lactose broth.

According to Grigoroff,¹ the bacillus bulgaricus attacks mannite, saccharose, maltose, and lactose, but not rhamnose, dulcitol, or sorbitol; according to Cohendy,² the bacillus attacks lactose, maltose, saccharose, levulose, and dextrose. In a later work, Bertrand and Duchacek³ state that this bacillus ferments dextrose, mannite, galactose, levulose, and lactose, but does not attack arabinose, xylose, sorbitol, maltose, and saccharose. The lack of agreement exhibited by these findings make them of doubtful value in the identification of this bacillus.

A similar and probably closely related organism is that isolated by Moro from infants' stools and referred to by him as the bacillus acidophilus. The similarity of behavior both biochemically, and as far as was known, culturally, of the acidophilic and bulgaricus groups led several writers, notably Rodella⁴ in 1908, and Heinemann and Hef-feran⁵ in 1909, to conclude that they are identical. Although a survey of the literature and a study of their properties leaves no doubt as to their common membership in the group of non-gas-forming, acid-resisting, lactose-splitting bacteria, there exist, nevertheless, well marked cultural differences that set them apart. These differences form the subject of this paper.

* Received for publication February 12, 1914.

1. *Rev. med. de la Suisse romande*, 1905, 25, p. 714.

2. *Compt. rend. Soc. de biol.*, 1906, 60, p. 558.

3. *Ann. de l'Inst. Pasteur*, 1909, 23, p. 402.

4. *Centralbl. f. Bacteriol.*, Abt. I, O., 1901, 29, p. 717.

5. *Jour. Infect. Dis.*, 1909, 6, p. 304.

The literature relating to the bulgaricus type of bacillus has been so often summarized that a repetition at this time seems unnecessary and the reader interested in that phase of the subject is referred to the articles of Heinemann and Hefferan,⁶ White and Avery,⁷ and Kuntze.⁸ The state of the literature with respect to the bacillus acidophilus is much the same as in the case of the bacillus bulgaricus; it is recognized as a fermentative organism but apparently little is known in regard to its action on different sugars. According to Moro,⁹ the bacillus acidophilus is an acid-resisting bacillus that grows poorly in the usual media and forms irregular colonies on agar. It grows at body temperature and also at 20 C. It is an acid former and coagulates milk. The bacillus acidophilus of Finkelstein has essentially the same cultural characteristics as Moro's bacillus with the exception that it does not coagulate milk, as stated by Lehmann and Neumann.¹⁰ Rodella¹¹ described a strain exhibiting branching and streptobacillus forms which gave colonies of different shapes on agar plates. Mereshkowsky,¹² who studied the distribution of this bacillus in the animal kingdom, recognizes two types of colonies formed by it, a round, compact, even-edged type, which he designates as Type 1, and one with a filamentous border, Type 2. He found no cultural difference between them. He made use of the following reactions in their identification: they live one to three days in 1 to 0.5 per cent acetic or lactic acid broth; they form the two mentioned types of colonies; they are Gram-positive, and they cause agar to become cloudy.

There seems to be no doubt of the wide distribution in nature of these bacteria and there is likewise no question as to their occurrence among the intestinal flora. Podajezky,¹³ who isolated them from bottle-fed and breast-fed infants, from a 10-year-old girl and from a man of 34, showed that the bacillus acidophilus exists in the intestinal tract regardless of age and variety of food. Mereshkowsky isolated it from a long series of animals ranging from mollusks to man.

The general characteristics of both the bulgaricus and acidophilus types of bacilli may be summarized as follows: They are bacilli of varying lengths, occur singly or in chains or threads and develop under both aerobic and anaerobic conditions. Typically they stain Gram-positive but old cultures may be Gram-negative. They produce acids from carbohydrates but do not form gas. They do not form spores. They survive and multiply in media containing considerable amounts of acid. Colony formation, the coagulation of milk, and action on carbohydrates are considered in detail further along.

Since Heyman in 1898 used acetic acid in dextrose broth in isolating a bacillus of the acidophilic type, many authors have followed his example and in this investigation a medium of the same general composition has been used, viz.,

6. *Ibid.*

7. *Centralbl. f. Bacteriol.*, I, O., 1910, 25, p. 161.

8. *Ibid.*, II, 1908, p. 161.

9. *Wien. klin. Wchnschr.*, 1900, 5, p. 114.

10. *Bacteriologie*, München, 1907.

11. *Centralbl. f. Bacteriol.*, I, O., 1901, 29, p. 717.

12. *Ibid.*, 1905, 39, p. 380.

13. Quoted by Mereshkowsky.

meat-peptone broth containing 2 per cent. glucose and 0.3 per cent. acetic acid. A little of the substance under investigation was seeded into a tube of the broth and incubated for forty-eight hours. One-half c.c. of this culture was then seeded into another tube, and again incubated. After a third seeding and incubation, a loopful of the broth was streaked on the surface of an agar plate. The triple seeding was made for the purpose of eliminating bacteria other than those of the type desired. This is the procedure recommended by Kendall.¹⁴ The agar used for plating was of the usual kind containing 1.5 per cent. of agar and 2 per cent. dextrose, but with no adjustment of its acidity. The growth obtained on this agar was fully equal to that on dextrose whey agar. Later the procedure recommended by Salge¹⁵ was adopted and 0.2 per cent. of sodium oleate added to this medium. As this oleate agar gave a better growth than the plain glucose agar described above, it was used throughout the latter part of the work. Its reaction varied from 0.8 to 1 per cent. The agar plates were incubated for forty-eight hours before the colonies were picked off. In a series of about 100 isolations only four gas formers were found; streptococci were few and aside from these and an occasional growth of yeasts, none but acid-resisting bacteria grew on the plates. The sources of material are given in Tables 1 to 3.

Aside from milk, the only medium used in the differential tests was unneutralized meat-peptone sugar-free broth with the addition of various carbohydrates to the amount of 2 per cent. The initial reaction of the broth varied from +2.2 to +2.8 per cent. Since this variation could not have affected the activity of the bacteria to an appreciable extent, and since the composition of the original broth could not be controlled, nor could a slight degree of hydrolysis be prevented during sterilization and incubation, the various lots of broth were considered in close enough accord for the purpose of this investigation. The broth was sterilized in bulk in the Arnold sterilizer and care was taken to prevent more than a very slight hydrolysis of the carbohydrate. The medium was then tubed in 9 c.c. amounts, and after a preliminary incubation test for sterility the tubes were seeded with a loopful of a forty-eight hour culture of the organisms to be examined, and incubated for five days at 37 C. Five c.c. of each culture were then titrated in the cold against N/10 NaOH, using phenolphthalein as an indicator. It will be observed that this medium is in every sense a "usual" one and it is difficult to see why it has not been used before for the purpose of differentiation. It is true that in the case of organisms of the *bulgaricus* type visible growth may not appear before forty-eight hours, but it is likewise true that in the presence of suitable sugars there is a measurable degree of fermentation at or before this time. Cultures in this broth have remained viable for a month or longer when kept in the ice chest.

Milk was tubed in 10 c.c. amounts and incubated for a period of 10 days. When clotting occurred the contents of the tube were worked up into a smooth mass and care taken to avoid air bubbles in pipetting the medium for titration. In Tables 1, 2 and 3 Mereshkowsky's terms have been used to indicate the type of colony formed, and the bacilli themselves are classified according to their reaction to Löffler's stain as suggested by White and Avery. In these tables the acid is calculated on the basis of 94 per cent. lactic and 6 per cent. volatile acid. These are the values determined by Heinemann and Hefferan.¹⁶

14. *Jour. Med. Research*, 1910, 22, p. 153. In this article Kendall introduces the name "aciduric" to take the place of "acidophilic."

15. *Jahrb. f. Kinderh.*, 1904, 59, p. 309.

16. *Jour. Infect. Dis.*, 1909, 6, p. 304.

Table 1 shows that the bacteria of this group all form colonies that correspond to Mereshkowsky's Type 2. Milk was coagulated as a rule before twenty-four hours at 37 C., and there was a large production of lactic acid during the six-day period. The action on maltose was so slight that it may be regarded as negative, and in one case there is a distinct reduction in acidity. With saccharose there was a slight acid production in two instances and a marked one in a third. In the column headed "Type of *B. Caucasicum* (White and Avery)" the strains are classified according to their reaction to Löffler's methylen blue. According to these authors those that are rapid fer-

TABLE 1.
FERMENTATIVE ACTIVITY OF BACTERIA OF GROUP 1

Culture	Source	Type of Colony (Mereshkowsky)	Type of <i>B. Caucasicum</i> (White and Avery)	Total Acidity Percentage of Normal Acid Six Days in Milk	Percentage of Fixed Acid in Terms of Lactic Acid	Percentage of Normal Acid in Maltose Broth, Five Days	Percentage of Normal Acid in Saccharose Broth, Five Days
Museum	Amer. Museum Nat. His.	2	A	26.3	2.198	0.0	0.1
SK.	Fairchild	2	A	25.3	2.114	0.0	0.0
580	Nat. Hist.	2	A	26.5	2.215	0.0	0.1
582	Nat. Hist.	2	A	25.7	2.147	0.0	2.4
624	Nat. Hist.	2	A	20.9	1.747	0.0	0.0
Bulgar ₁	Milk (fermented)	2	B	13.0	1.087	0.2	0.2
Bulgar ₂	Milk (fermented)	2	B	14.8	1.237	0.1	0.1
Zoo	Milk (fermented)	2	B	14.4	1.199	0.0	0.0
B.B.	Drug Store	2	A	25.4	2.122	0.0	0.2
Massolin	Lederle	2	A	11.4	0.953	-0.4	0.4
Bulgara	Tablet	2	A	28.0	2.319	0.2	0.2
Bacilline ₃	Tablet	2	A	24.6	2.056	0.0	0.6

menters forming inactive lactic acid and staining solidly are called Type A, while those bacilli that are less active, forming levorotary acid and exhibiting purplish granules when stained are termed Type B. In the present work only the staining reaction was made use of in separating these two types. Culture "Massolin" showed a lower acid production than any of the Type B strains, though this is the only exception. It will be noticed that all three of the saccharose fermenters are of Type A.

Microscopic observation showed the organisms of this group to be Gram-positive long bacteria. In older cultures Gram-negative forms occur, and in one instance the buds and stems mentioned by White and Avery were found. Branch and string formations were noted, but there were no short streptobacilli in this group.

The bacteria included in Table 2 form both types of colonies. Milk is coagulated in from forty-two hours to six days. The lactic acid production varied from 0.201 per cent. to 1.304 per cent., the maximum acidity here being about one-half as great as the maximum of Group 1. As the table shows, the bacilli were isolated from various sources. Both long and streptobacilli occurred, and with the exception of culture "S $1\frac{1}{2}$ " in maltose and saccharose, and culture "Jam" in saccharose, the acid production was marked. Although the limit for clotting was set at six days most of the strains included in the above table caused solidification of the milk before ninety-six hours. In no instance was much whey expressed from the clot.

TABLE 2.
FERMENTATIVE ACTIVITY OF BACTERIA OF GROUP 2

Culture	Source	Type of Colony (Mereshkowsky)	Type of Bacillus	Total Acidity Percentage of Norm in 1 Acid Six Days in Milk	Percentage of Fixed Acid in Terms of Lactic Acid	Percentage of Normal Acid in Maltose Broth, Five Days	Percentage of Normal Acid in Saccharose Broth, Five Days
K	Typhoid stool	1	Streptobacillus	12.5	1.045	2.5	3.6
Jam	Saliva	1	Long	9.1	0.753	4.6	0.2
G	Typhoid stool	1	Long	8.5	0.710	8.0	10.5
G ₁	Putrefactive stool	1	Long	4.2	0.350	4.3	2.0
R _{2a}	Normal stool	1	Streptobacillus	12.8	1.070	6.0	5.0
HF ₉	Typhoid stool	2	Long	8.0	0.669	4.2	1.2
HF ₇	Typhoid stool	1	Long	5.6	0.468	8.2	5.8
HF ₃	Typhoid stool	2	Long	4.0	0.334	1.2	1.2
S $1\frac{1}{2}$	Typhoid stool	2	Long	8.6	0.719	0.3	0.2
Boh ₂	Normal stool	1	Streptobacillus	11.8	0.986	0.8	0.7
Boh	Normal stool	2	Long	3.6	0.301	1.1	2.0
DGC	Typhoid stool	2	Streptobacillus	15.6	1.304	3.5	3.1
L	Typhoid stool	1	Long	5.8	0.485	5.1	4.0
Mt.	Normal stool	1	Long	6.1	0.510	8.4	3.6
Z ₂	Fermented milk	2	Long	3.6	0.301	1.9	1.9
Sim	Normal stool	1	Long	10.4	0.869	0.6	1.2
Fly	Intestine of fly	2	Long	2.4	0.201	1.2	1.7
DGC ₂	Typhoid stool	1	Long	13.2	1.102	6.0	4.8
B	Typhoid stool	1	Streptobacillus	2.0	0.167	4.4	0.7
R ₂	Normal stool	1	Streptobacillus	11.4	0.953	4.9	6.0
HF ₄	Typhoid stool	1	Long	4.0	0.334	3.4	1.2

It will be seen on reference to the table that in milk both the most and least active strains are streptobacilli and that these form both types of colonies. Both long and streptobacillus forms showed bipolar staining organisms with Löffler's stain. Although the time of clotting is of some value in differentiating the members of this group from those of Group 1, the essential difference is their active splitting of maltose, and to a less extent that of saccharose.

In this group (Table 3) we have the bacilli that do not clot milk, and the acid production in no instance rises above 2.3 per cent. normal. Both types of bacilli and both types of colonies were present.

The tables show that all of the strains investigated fell naturally into three groups according to their action on milk or maltose broth. The organisms of Table 1 were in some instances pure cultures, in others isolated from lactic acid bacilli tablets or from fermented milks. None of the bacteria isolated from other sources were of this type. The members of Group 2 sometimes showed an acidity in milk nearly

TABLE 3.
FERMENTATIVE ACTIVITY OF BACTERIA OF GROUP 3

Culture	Source	Type of Colony (Mereshkowsky)	Type of Bacillus	Total Acidity Percentage of Normal Acid Six Days in Milk	Percentage of Fixed Acid in Terms of Lactic Acid	Percentage of Normal Acid in Maltose Broth, Five Days	Percentage of Normal Acid in Saccharose Broth, Five Days
F	Typhoid stool	1	Long	0.3	0.025	8.8	10.4
S	Putrefactive stool	1	Long	2.3	0.192	1.8	1.8
Kor	Typhoid stool	1	Long	1.3	0.109	6.0	8.0
C	Typhoid stool	1	Streptobacillus	0.3	0.025	9.4	6.6
Milk	Pasteurized milk	1	Long	0.7	0.059	8.8	8.4
D	Normal stool	1	Long	0.6	0.050	7.3	9.3
P	Typhoid stool	1	Long	1.0	0.084	5.4	0.5
HF	Typhoid stool	1	Long	0.2	0.017	6.5	7.7
CF	Typhoid stool	2	Long	1.8	0.151	1.2	4.1
Te	Saliva	1	Long	0.1	0.008	9.2	4.1
Wil	Saliva	1	Streptobacillus	0.7	0.059	9.8	8.5
Rat	Rat feces	1	Long	0.4	0.033	10.3	10.3
CF ₂	Typhoid stool	1	Long	0.0	0.0	10.4	8.9
HF ₈	Typhoid stool	1	Long	0.8	0.067	2.0	0.2
G ₂	Putrefactive stool	2	Long	0.0	0.0	7.3	6.6
Wrgt	Normal stool	1	Long	0.8	0.067	5.1	6.7
Wrgt ₂	Normal stool	1	Long	1.2	0.101	5.2	6.4
Tas	Normal stool	1	Long	1.2	0.101	8.8	0.0
Bacilline	Tablet	2	Long	0.0	0.0	3.4	4.0
R	Normal stool	1	Long	1.0	0.084	1.8	4.1
O. T.	Normal stool	2	Long	2.2	0.184	1.4	1.0

as great as that of some of the organisms of Group 1, but they were sharply set off from it by their action on maltose. In Group 3 milk is not coagulated, and the action on both maltose and saccharose on the whole is greater here than in either of the preceding groups.

It would appear that the bacillus *bulgaricus*¹⁷ forms but one type of colony while the bacteria of the "acidophilus" variety form two. On what the colony variation in the latter depends the writer does not attempt to say, but neither the opinion of Sandberg,¹⁸ that it is due

17. The writer is indebted to Prof. C. E. A. Winslow, American Museum of Natural History, New York City, for the pure cultures of the bacillus *bulgaricus*.

18. *Ztschr. f. klin. Med. Berl.*, 1903, 51, p. 80.

to increased acid production, nor that of White and Avery,¹⁹ that it is dependent on the composition and consistency of the medium, seems to explain the variation.

Tested in milk at room temperature, members of these groups showed widely varying degrees of activity. Only one culture in Group 1 clotted the milk in fourteen days. Group 2 showed greater activity, some strains clotting in six or seven days. In Group 3 culture "Milk" clotted in fourteen days though it did not do so at 38 C.

Tables 4, 5 and 6 show the effect of these bacteria on a series of five carbohydrates after five days at 37 C. For convenience the columns showing the action on maltose and saccharose are reproduced in these tables.

TABLE 4.
THE ACTION OF GROUP 1 ON CERTAIN CARBOHYDRATES

Culture	Percentage of Normal Acid in Dextrose Broth	Percentage of Normal Acid in Lactose Broth	Percentage of Normal Acid in Saccharose Broth	Percentage of Normal Acid in Maltose Broth	Percentage of Normal Acid in Levulose Broth	Percentage of Normal Acid in Mannite Broth
Museum	5.7	5.9	0.1	0.0	0.5	0.4
SK	16.0	13.9	0.0	0.0	2.4	0.2
580	7.4	7.5	0.1	0.0	2.3	—0.2
582	5.9	3.1	2.4	0.0	0.9	0.2
624	5.4	0.9	0.0	0.0	0.7	—0.2
Bulgara	0.4	3.6	0.2	0.2	0.1	0.0
Bulgara ₂	1.3	0.8	0.0	0.1	—0.4	0.2
Zoo	4.5	2.4	0.0	0.0	—0.3	0.2
B. B.	2.1	2.0	0.2	0.0	0.0	0.4
Massolin	8.6	5.8	0.4	—0.4	0.2	—0.2
Bulgara	1.8	1.6	0.2	0.2	0.2	0.3
Bacilline ₂	8.4	3.4	0.6	0.0	0.0	—

It is evident from this table that these strains have a decided preference for dextrose and lactose; in only two or possibly three instances was saccharose attacked. Seven of the twelve strains did not attack levulose, and mannite showed only a very slight fermentation. In those carbohydrates that were but slightly acted on there were occasional instances in which there was a reduction in the acidity. Duplicate tests confirmed this and the same effect occurred elsewhere as can be seen by reference to the tables. Table 5 shows the results with the organisms of Group 2 on the same sugars.

19. *Centralbl. f. Bacteriol.*, 1910, 25, p. 161.

A comparison of this table with Table 2 shows that the acid production in glucose broth in most instances compares very well with that in milk. Lactose, saccharose, and levulose are utilized by all or nearly all of its members, and the differential value of maltose is strongly brought out. Culture "S $1\frac{1}{2}$ " is the only organism whose place in this group may be questioned. It was placed here because of its rather slow coagulation of milk—seventy-two hours at 38 C. It is interesting to note that all of the mannite fermenters fall within this group, the sole exception being culture "Wil" in Group 3.

TABLE 5.
THE ACTION OF GROUP 2 ON CERTAIN CARBOHYDRATES

Culture	Percentage of Normal Acid in Dextrose Broth	Percentage of Normal Acid in Lactose Broth	Percentage of Normal Acid in Saccharose Broth	Percentage of Normal Acid in Maltose Broth	Percentage of Normal Acid in Levulose Broth	Percentage of Normal Acid in Mannite Broth
K	12.2	8.4	3.6	2.5	11.3	4.1
Jam	7.0	6.7	0.2	4.6	10.1	1.7
G	7.7	6.7	10.5	8.0	10.0	1.9
G ₁	0.5	1.4	2.0	4.3	8.2	1.8
R _{2a}	7.1	5.0	6.0	3.4	9.1	3.8
HF ₉	3.3	1.2	1.2	4.2	7.1	1.4
HF ₇	7.5	5.8	5.8	8.2	8.5	0.0
HF ₄	4.4	2.4	0.2	3.4	3.3	2.0
HF ₃	1.0	0.2	1.2	1.2	0.7	0.6
S $1\frac{1}{2}$	1.4	1.2	0.2	0.3	0.6	1.0
Boh ₂	9.0	8.0	0.7	0.8	10.0	2.8
Boh	2.8	1.8	2.0	1.1	1.2	0.0
DGC	10.0	8.4	3.1	3.5	10.4	2.0
L	5.8	4.0	4.0	5.1	5.8	2.6
Mt.	3.9	7.7	3.6	8.4	9.7	0.8
Z ₂	0.8	3.2	1.9	1.9	0.6	0.0
Sim	8.2	8.0	1.2	0.6	11.0	1.8
Fly	2.4	1.6	1.7	1.2	1.4	0.0
D.GC ₂	7.9	4.6	4.8	6.0	9.6	2.6
B	8.6	4.4	0.7	4.4	8.4	1.4
R ₂	7.3	4.4	6.0	4.9	9.1	3.8

Table 6 contains the members of Group 3. Although thirteen out of twenty of these strains showed marked acid production in lactose broth the acid formed in milk was very much less.

From the experiments detailed it seems that the bacilli of the bulgaricus type differ in some important essentials from those usually included under the term "acidophilus." To the writer it appears probable that the bacillus bulgaricus is a milk bacillus, and that its appearance in the intestine practically never occurs unless it has been ingested in enormous numbers. Bacteria of the acidophilic type, on

the other hand, are normal inhabitants of the digestive tube. The question of the survival of the *Bacillus bulgaricus* in the intestine in the light of its identification by means of the maltose reaction will be considered elsewhere.

In addition to the media mentioned above, cultivation of these bacteria was attempted in sugar-free broth and agar. With the exception of a very faint growth in the case of culture "Kor" of Group 3 none of the strains grew in sugar-free broth. The acidity of this broth was + 2.8 to phenolphthalein. On sugar-free agar, however,

TABLE 6.
THE ACTION OF GROUP 3 ON CERTAIN CARBOHYDRATES

Culture	Percentage of Normal Acid in Dextrose Broth	Percentage of Normal Acid in Lactose Broth	Percentage of Normal Acid in Saccharose Broth	Percentage of Normal Acid in Maltose Broth	Percentage of Normal Acid in Levulose Broth	Percentage of Normal Acid in Mannite Broth
F	10.4	9.8	10.4	8.8	12.5	0.0
S	3.2	1.5	1.8	1.8	3.1	-1.2
Kor	4.6	7.9	8.0	6.0	8.1	0.0
C	3.4	11.5	6.6	9.4	3.1	0.1
Milk	4.1	0.9	8.4	8.8	7.9	0.1
D	6.6	4.6	9.2	7.3	8.4	-0.4
P	4.0	3.2	0.5	5.4	7.2	0.4
HF	7.8	0.2	7.7	6.5	8.0	0.0
CF	1.0	1.0	1.7	1.2	4.4	0.0
Te	4.4	5.7	4.1	9.2	13.8	0.2
Wil	6.0	6.3	8.5	9.8	15.0	3.2
Rat	10.1	6.6	10.3	10.3	6.1	0.2
CF ₂	11.0	11.0	8.9	10.4	2.8	0.0
G ₂	7.9	6.8	6.6	7.3	1.1	0.4
Wrgt	5.1	4.0	5.8	5.1	6.7	0.2
Wrgt ₂	0.9	5.6	2.4	5.2	6.4	0.0
Bacilline	0.6	1.4	4.0	3.4	2.6	0.3
O. T.	0.4	1.0	1.0	1.4	1.0	0.2
Tas	8.5	6.4	-0.2	8.8	1.5	0.4
Ri	1.8	0.6	4.1	1.8	2.0	-0.2

different results were obtained. Although after seventy-two hours the members of Group 1 showed no growth, those of Groups 2 and 3 showed fair development in every instance, though the colonies were small. This agar had a reaction of + 1.

In order to study the production of cloudiness in agar, puncture cultures were made in oleate agar. This clouding was not a constantly occurring characteristic, though it appeared in all three groups. In Group 1, in those instances in which it did happen, the cloudiness appeared after seventy-two hours' incubation in the lower part of the tube and in the region of the puncture, spreading outward and upward

until the end of six days when the agar was uniformly clouded. Among the members of Groups 2 and 3 the cloudiness started earlier and spread evenly. Although a good growth was obtained in every instance, some strains showed not the slightest cloudiness even after six days at 37 C.

In addition the effect of these bacteria on raffinose was studied. This sugar was attacked in a greater or less degree by all of the bacilli of Groups 2 and 3 but those of Group 1 did not act on it.

CONCLUSIONS

From the facts brought out in this investigation the writer is of the opinion that there are three varieties of bacilli in the division of acid-resisting bacteria, at least two of which are of constant occurrence among the fecal organisms:

Variety A, which clots milk but has no action on maltose; Variety B, which clots milk and ferments maltose; and Variety C, which ferments maltose but does not clot milk.

Bacilli of the type of the *bacillus bulgaricus* may be cultivated in unneutralized meat-peptone broth containing a suitable carbohydrate.

In broth the *bacillus bulgaricus* does not ferment maltose, and may be differentiated from the other acid-resisting organisms by this characteristic.

The writer acknowledges his indebtedness to Dr. J. C. Torrey for valuable suggestions.

PURPURA ASSOCIATED WITH BACILLUS MUCOSUS IN THE BLOOD *

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Reports of bacteriemia due to members of the bacillus mucosus group of organisms, in which the diagnosis has been verified by blood-culture during life, are comparatively infrequent in the literature. At autopsy, however, cultures have been reported quite frequently in which the evidence was in favor of such infection. In the case here reported capsulated bacilli were twice recovered from the blood and the urine during life, as well as from the heart's blood, various organs, and urine at autopsy. Our case is also of interest in its clinical course, the development of the blood infection from the urethra and the late onset of the purpura.

The term bacillus mucosus capsulatus employed by Fricke for the group of bacteria here referred to, is the one which we prefer rather than bacillus pneumoniae (Friedländer), since the former is descriptive of one of the characteristics common to all the members of the group, is indicative of no particular disease, and is broad enough to include the many Gram-negative capsulated bacilli which show common biological and cultural characters. There has been and still is confusion in the nomenclature, as shown by the long list of names applied to bacteria, which undoubtedly are members of the mucosus group. The general characteristics of the group are as follows: The bacilli occur as short plump rods, singly or in pairs, often coccoid, while long and even thread-like forms may develop and under certain circumstances fairly long chains occur. No spores are formed. Motility is not observed. Capsules are produced in most media, the addition of serum favoring their development. Growth is luxuriant on all media, aerobic conditions being more favorable than anaerobic. A mucus-like substance is formed in the cultures. Coagulated serum, casein and gelatin are not liquefied. Indol is generally not formed in peptone solution. Fermentation to acid and gas of certain carbohydrates is characteristic of the group, the members differing in the carbohydrates they attack,

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and in the degree of fermentation. Marked tenacity of life is exhibited under ordinary conditions.

Pathogenicity for man and the laboratory animals is somewhat variable, but usually present.

In the classification of the organisms belonging to this group we have found that all the Gram-negative capsulated bacilli, which we have obtained in the laboratory from a variety of sources, come under one or the other of three types, as described by Perkins¹ and others.

This led us to adopt the following practical classification, based on the fermentation of certain carbohydrates: (1) The bacillus lactis aërogenes (Escherich), which possesses the most marked power of fermentation, producing acid and large amounts of gas in dextrose, lactose and saccharose broth. It has been our experience to find this member most frequently. (2) The bacillus acidi lactici (the bacillus duodenale of Ford²) possesses in a lesser degree the power of fermentation. It produces acid and gas in dextrose and lactose broth, but fails to attack saccharose. It produces very much less gas than the bacillus lactis aërogenes. (3) The bacillus pneumoniae (Friedländer) ferments dextrose and saccharose to acid and a small amount of gas. In our experience these organisms, when thoroughly studied, produce a late and slight fermentation of lactose. Litmus milk is not coagulated. This micro-organism is found much less frequently than either of the other forms.

The difficulties which have arisen in establishing a classification of the members of the mucosus group can, in a great measure, be traced to incomplete examination, and the failure to recognize the fact that organisms which have the ability to ferment carbohydrates may lose the power in whole or in part through changes in environment or unfavorable conditions (Perkins³). The fermentation reactions are no more variable than in the bacillus coli recently isolated from water. In making this classification we would not exclude those indefinite organisms reported in the literature, but we would state that they belong, in all probability, to the degenerated or other types referred to.

As a cause of disease, the capsulated Gram-negative bacilli have been recognized in a great variety of pathological conditions, the discussion of which, at this time, would lead us too far afield and we would rather confine our remarks to those points which chiefly concern the

1. *Jour. Infect. Dis.*, 1907, 4, p. 951.

2. *Stud. Roy. Victoria Hos., Montreal*, 1903, p. 1.

3. *Jour. Infect. Dis.*, 1907, 4, p. 951.

case reported in this paper. However, we would here call attention to the occurrence of Gram-negative capsulated bacilli in conditions involving the respiring tract, accessory sinuses, middle-ear, gastro-intestinal tract and not infrequently the genito-urinary tract.

In reviewing the literature we find that the presence of a definite bacteriemia due to the bacillus mucosus is comparatively rare. Wolff¹ was able to collect from all available literature prior to 1909, 39 cases, the organism recovered being referred to as the bacillus pneumoniae (Friedländer). In 11 of these cases the organism was recovered from blood before death. In the remaining cases, however, he states that the clinical and pathologic picture before death was so definite that they might be classified with the cases of true bacteriemia without comment. Nineteen of the 39 cases had their primary focus in the lung, 17 were of cryptogenic origin, 5 arose in the liver and gall ducts, 5 came from the urogenital tract, 2 from otitis media, and 1 from a phagedenic ulcer. In 13 of the 39 cases, metastases were found in the liver (5 times), in the kidney (4 times), in joints (3 times), in the ear (2 times), in the thigh (1), and in the sternocleidomastoid muscle (1). Two of the above cases recovered.

Hirschbrook and Ziemann⁵ report a fatal case in which the bacillus lactis aërogenes was isolated at autopsy from the heart's blood and different viscera. Owing to the clinical manifestations and the recovery of the bacillus typhosus from the stools, this case was first looked on as one of ordinary typhoid fever. Subsequently, however, the symptoms became very acute with the development of purpuric spots and a second examination of stools failed to show presence of the bacillus lactis aërogenes widely disseminated in the body. Diffuse petechial hemorrhages were present. From the results of examination after death it was maintained that the condition was one of the bacillus mucosus capsulatus bacteriemia developing secondarily to typhoid.

Closely associated with the systemic infection by the bacillus mucosus is the development of petechial hemorrhages, not only on the skin surfaces, but also in various viscera, and on the lining membranes of the body cavities. In a review of 10 cases, Abel and Hallwachs⁶ showed the frequency of occurrence of hemorrhagic purpura associated with the bacillus mucosus in the blood. From the literature we have found that the petechial hemorrhages seem to arise at the time when the patient is overwhelmed with infection, 5 or 7 days prior to death. These hemorrhages make their appearance as an erythematous rash, later developing a more bluish and finally a deep cyanotic character.

It has not infrequently been found that the bacteriemia due to the bacillus mucosus has originated from the genito-urinary tract. Chiari has reported a case of ascending infection of the genito-urinary tract which at autopsy showed cystitis, prostatic and renal abscesses, endocarditis, infarct of the spleen, and purulent meningitis. Howard⁷ has also reported a case of bacteriemia arising from the genito-urinary tract showing chronic cystitis, renal abscess, and peritonitis. It is worthy of note that localized infections simulating chronic gleet may result from an infection by the capsulated Gram-negative bacilli. A case of chronic urethritis reported by Avery,⁸ and another with urinary fistula from which

4. Beiträge zur Pathologie der durch den Bazillus Friedländer erzeugten Sepsis, Dissertation, Leipzig, 1909.

5. *Cent. f. Bakteriöl., orig.*, Abt. 1, 1913, 70, p. 281.

6. Kolle and Wassermann, *Handb. d. Path. Mikroorg.*, 1913, 6, p. 515.

7. *Jour. Exper. Med.*, 1899, 4, p. 149.

8. *Standard Methods of Water Analysis*, 1912, p. 84.

the organisms isolated had characters of the type of the bacillus lactis aërogenes was reported by White.⁹ Bernstein isolated capsulated Gram-negative bacilli from an acute epididymitis, and also from a purulent orchitis. Schenk and Weltman isolated capsulated Gram-negative bacilli from the inflamed fallopian tubes. Allen¹⁰ reported a case of gleet due to the bacillus pneumoniae (Friedländer) which, when treated by autogeneous vaccine, was followed by a satisfactory recovery. As an etiologic factor of inflammatory processes in man we find that the bacillus lactis aërogenes had been most frequently isolated.

Heyse,¹¹ Wildholz¹² and Schnitzer¹³ each reported a case of pneumaturia due to the bacillus lactis aërogenes, while Trumpp¹⁴ found one such infection in 29 cases of cystitis in children. In none of these cases did a generalized infection occur, but the symptoms were at times quite severe.

Recently Leutscher¹⁵ reported 2 cases of the bacillus lactis aërogenes infection of the bladder and commented on the infrequent presence of the organism as a cause of cystitis. His cases are interesting. In the first, a woman developed cystitis during the second month of pregnancy and subsequently infected her husband. Both suffered urethritis and cystitis from which the bacillus lactis aërogenes was isolated.

The case which we desire to report was an Italian of 42 years. He was admitted to the Mercy Hospital, April 1, 1913, under the service of Dr. J. P. Griffith. He complained chiefly of painful, frequent, and difficult urination. He stated that the present illness began Jan. 24, 1913, when he suffered a severe chill as well as some bladder distress. He was treated by a physician, and sounds were passed into the urethra. His chills then became periodic, recurring once or twice a week. The patient always recovered completely from effects of each chill and he was able to go about during the intervals.

The patient was slightly emaciated and rather anemic. Considerable tenderness over the left lobe of prostate with slight enlargement. The passage of sounds showed an obstruction in the posterior urethra.

The patient had had the usual diseases of childhood and recovered without complications. He suffered an attack of malaria at 19 years and an attack of pneumonia at 32 years. He had been married 15 years and migrated to America 5 years ago. He developed gonorrhea 20 years ago, which continued in a chronic state many years. Ten years ago he claimed to have had a condition similar to the present from which he fully recovered.

The circulatory, respiratory, and digestive systems appeared normal. The urine showed the presence of a slight amount of albumen and some leukocytes. Gonococci could not be demonstrated.

April 3.—Two hours after first passage of sounds, the patient suffered a severe chill and fever, temperature 105.4 F. The patient remained in bed the following day and appeared quite well. Temperature and pulse normal.

April 9.—Eight days after admission the stricture was forcibly dilated under anesthesia, which was followed in a few hours by a severe chill and high fever, 105.6 F. The following day the patient was again sufficiently well to leave his bed.

9. *Ibid.*

10. *Vaccine Therapy*, 1910, p. 127.

11. *Ztschr. f. klin. Med., Berl.*, 1894, 24, p. 130.

12. *Cor.-Bl. f. schweiz. Aerzte*, 1901, 21, p. 683.

13. *Internat. Klin. Rundschau*, 1894, 8, p. 265.

14. *München. med. Wchnschr.*, 1896, 43, p. 1008.

15. *Bull. Johns Hopkins Hosp.*, 1911, 22, p. 360.

April 17.—Sounds were again passed and the patient again suffered a severe chill, with a fever of 103.4 F., from which he fully recovered on the following day. The urethra at this time was quite patent and permitted the passage of a 28 F. sound. Examination of urine at this time showed a slight amount of albumen with a few leukocytes, while in culture the bacillus lactis aërogenes was isolated.

Following the chill on April 17, the patient recovered except for a general muscular soreness and several painful points, especially about the limbs and shoulders. The patient had been receiving ordinary urinary antiseptics. On May 3 he complained of a burning sensation in the bladder and penis, especially during urination.

May 15.—Sounds were again passed into the urethra. No evidence of stricture was found at this time. Shortly following the passage of sounds (37 F.) the patient suffered severe chills and fever of 104.6 F. From this date on the temperature persisted with frequent chills and fever. The patient's general condition was not greatly affected, save for great lassitude.

May 22.—Following another chill a blood culture was taken. The bacillus lactis aërogenes was isolated.

Blood examination:

Hemoglobin	70 per cent.	Polynuclears	87 per cent.
Red cells	4,098,000	Eosinophils	1 per cent.
Leukocytes	68,000	Large lymphocytes....	3 per cent.
		Small lymphocytes....	8 per cent.
		Transitional forms....	1 per cent.

May 26.—Since last passage of sounds the patient had a septic temperature with frequent chills; appetite greatly impaired and evidence of emaciation and anemia appearing; pains in limbs and continuous sharp pain over precordium, but examination of chest and heart showed no lesion.

The high temperature continued, the pulse of good volume, not above 120. The chest wall showed petechial hemorrhages, hip and limbs a mottled erythematous condition.

May 29.—Examination of prostate revealed a great tenderness, but only slight enlargement. After thoroughly cleansing urethra, a culture of the bacillus lactis was obtained of the prostatic secretion by massaging the posterior urethra.

May 30.—The general condition was quite alarming, with vomiting for past few days.

May 31.—The temperature was still very high; intense burning and dryness in the throat. A catheterized specimen of urine gave a pure culture of the bacillus lactis aërogenes. Hemorrhagic spots became darker and more diffuse. There was a slight amount of albumen in urine, but no leukocytes. Red cells, 3,870,000; hemoglobin, 65 per cent; leukocytes, 9,700.

June 1.—Blood culture was found to contain the bacillus lactis aërogenes.

June 6.—For three days the patient appeared very toxic and although rational, he was very nervous. Pain in the chest was intense and continuous. He was unable to swallow much food but had an intense thirst. His temperature was high, but the pulse was not above 130 per minute; the skin showed a diffuse mottling of petechial hemorrhages considerably darker and more bluish than when first observed.

June 7.—Death.

It is evident that we were dealing with an infection due to the bacillus lactis aërogenes. Blood cultures on two different days gave a pure growth of the bacillus lactis aërogenes. The cultures were made in 1 per cent. dextrose serum broth and at the end of 24 hours the blood clot was blown up, floating in the medium with many white vertical, candle-like growths.

Cultures of the bacillus lactis aërogenes were also recovered from the prostatic urethra and the urine. From the bacteriologic evidence the genito-urinary tract appeared to be the point of entrance for the development of the bacteriemia.

Autopsy twenty-four hours after death (Dr. O. Klotz.)—The surface of the body rather pale, the face yellowish, the teeth poor, mucous membranes pale. Over the neck, chest, abdomen, thighs and arms was a diffuse purpuric rash, which, for the most, was pinhead in size, but which occasionally showed small hemorrhagic blotches, the largest 1.2 cm. in diameter. The face, hands, and legs below the knees, showed none of these spots. The rash was also seen on the back where postmortem lividity was well developed.

There was a single old adhesion near the apex of the left lung and some diffuse adhesions over the diaphragmatic surface of the right lung. The pericardium contained about 150 c.c. of turbid fluid with many lymph flakes; the surface dull and granular with a single calcareous nodule near the apex of the left lung. The heart weighed 550 G. The right ventricle was large; projecting into its upper part just below the pulmonary valve and lying behind the insertion of the tricuspid valves on the septum was a mass, 4 cm. in diameter, the muscle over it of a yellow necrotic color with hemorrhages; the muscle of the right ventricle otherwise pale and cloudy. The pulmonary and tricuspid valves normal. Throughout the muscle of the left ventricle many yellow spots with hemorrhagic streaks or dots. The papillary muscles showed yellow mottling. The coronary arteries over the surface of the left ventricle very prominent, with definite chains of little nodules in some of them. The mitral valve quite healthy, save for a single, small deposit of recent fibrin. The aortic orifice over half its extent obliterated by a massive thrombotic deposit resting on the heart muscle and the aortic valve in front of the posterior coronary artery filling the corresponding sinus; this mass, which completely obliterated half of each of the adjoining valves at this point, was the size of a small walnut. The vegetation rested on that portion of the heart muscle which was observed to project into the right ventricle. The myocardium just below the aortic ring was much thickened and appeared partly necrotic; in cutting it a small necrotic cavity or abscess was entered; the posterior coronary artery passed over the outside of this mass and was so compressed that its lumen was all but obliterated. When more closely examined, the mass was found to occupy a saccular dilatation of the lower portion of the posterior sinus of Valsalva which projected into the musculature of the septum and posterior parietes so that a protrusion had taken place into the septum and right ventricle.

The base of the aorta quite smooth and elastic, nodular plaques in the thoracic and abdominal portions.

The peritoneum, mesentery, and omentum dotted by small petechial hemorrhages, the majority of which were the size of a pinhead.

The gastric mucosa showed numerous petechial hemorrhages and near the pyloric ring were a number of the marked erosions of the surface.

Here and there in the small and large bowel submucosal hemorrhages were observed, at times 1 cm. in diameter.

Along the upper and anterior border of the spleen, which was large, was a yellow area 2.5 cm. in width, with a dark red center which extended into the organ as an infarct; the spleen substance easily broken, dark in color.

The kidneys large, capsule peeled readily; on the surface many small gray areas about the size of a pinhead; in the left kidney one larger yellow area surrounded by hemorrhagic zone; the cortex and medulla not well defined; the small gray dots observed on the cortex seen as fine gray lines on the cut surface.

The bladder small, walls somewhat thick, the mucous membrane everywhere dotted with bright red hemorrhages, the majority of which were of pinhead size. The prostate not enlarged, but showed some small purulent areas in its substance.

The principle results of the microscopic examination may be summarized by saying that there was a chronic and acute myocarditis and suppurative nephritis.

Culture from the heart blood, from the abscess in heart, from the pericardium, bile, contents of small intestines, and from the bladder, all gave the *bacillus mucosus*. From the abscess in the heart there was isolated also the *micrococcus tetragenus*.

Anatomical Diagnosis.—The *bacillus mucosus capsulatus* bacteriemia; infectious purpura; acute vegetative aortic, mitral and mural endocarditis, acute mycotic aneurysm of sinus of Valsalva, stenosis of aortic orifice; acute purulent myocarditis; hypertrophy and dilatation of the heart; acute serofibrinous pericarditis; chronic interstitial myocarditis; petechial hemorrhages of skin, pleura, pericardium, stomach, intestines, peritoneum, pelvis of kidney, ureter, and bladder; acute splenitis; splenic infarct; infarcts and multiple abscesses of kidneys; acute purulent prostatitis.

The organisms isolated from the blood, urine, and prostatic secretion before death and those from the blood and various organs at postmortem were carefully studied. They all showed the following characteristics: They appeared mostly in the form of short plump rods with rounded ends, although in some of the older cultures irregularly sized bacilli were seen forming long and short threads. From the milk agar plates, chains of short capsulated diplobacilli were seen. The bacilli were non-motile, definitely Gram-negative, and did not form spores. Capsules were easily demonstrated from the litmus milk and other media.

In plain broth at the end of twenty-four hours there was a slight cloud, which gradually increased, the broth becoming quite milky in appearance with a heavy white gummy sediment and the development of a thin shiny pellicle especially around the edges, forming a ring on the wall of the test tube. There was no odor.

Plain agar slant showed a shiny, luxuriant, well-defined streak at the end of twenty-four hours. This growth later became heaped up and porcelain-white in color, mucoid, watery, with the growth running down to the bottom. It was very viscous, drawing out in long strings with the platinum needle.

In gelatin stab cultures, the organism grew in a spreading white film on the surface, which did not become heaped up but remained quite flat. Along the line of the stab, fine granules were formed which later ran together and formed a compact streak. After considerable time there appeared fine outgrowths from the stab and the gelatin along the stab became quite cloudy. The gelatin was not liquefied and there was no browning after three months.

On potato slant the growth was shiny, cream colored, sticky and showed much gas production. The growth, after two weeks, became pale yellow.

There was no indol formed after four days in Dunham's peptone solution, tested by Ehrlich's paradimethylamidobenzaldehyde test.

Cultures on litmus milk showed marked acidity in twenty-four hours. On the second day there was firm coagulation with later reduction of the litmus, the clot separating from the clear whey. Capsules easily stained by Welch's method.

Dextrose, lactose, saccharose, mannite, and raffinose broths showed fermentation to acid and much gas in twenty-four hours. At the end of two weeks there was complete decolorization of the fuchsin indicator with absorption of a varying amount of the gas. Dulcitol was not attacked. Potato starch broth showed gas production, as did also deep potato starch agar.

On litmus milk agar plates (at the end of twenty-four hours) a spreading watery growth developed which ran down on the cover of the Petri dish. When it was lifted off, long strings of the mucoid growths were formed. At the end of twenty-four hours the medium was pale pink, and in several days the reaction became distinctly alkaline. This reaction was limited to the region of the colonies.

The organism is undoubtedly the *bacillus lactis aërogenes* of the *mucosus* group. We were unable to list it under the classification in "Standard Methods for Water Analysis."

SUMMARY

Clinically, the case was a progressively severe bacteriemia in which cultures of the *bacillus lactis aërogenes* were obtained during life from the blood, urine and prostatic secretion.

Purpura, although a late manifestation, was the result of this infection.

On account of the systemic reactions after each surgical interference of the urethra, we feel convinced that this was the point of entrance of the infection into the blood-stream.

Probably infection of the genito-urinary tract by bacteria of the *mucosus* group is more common than realized.

STUDIES ON THE SANITATION OF SWIMMING POOLS *

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The present investigation was undertaken to inquire into the sanitary conditions existing in swimming pools, and to study methods of improving these pools if defective, without altering too greatly their equipments or modes of administration. In addition to this, it has been our purpose to determine as accurately as possible, standards in construction, equipment, and management which we think should be required of such establishments in the future.

The problem of the sanitary swimming pool has assumed considerable importance. The dangers of bathing in polluted water have been pointed out by many authors, and numerous plans for overcoming these dangers have been suggested. Many municipalities have established public baths and swimming pools. Swimming pools have been installed by private individuals, as well as by colleges and universities, secondary and elementary schools, clubs, steamship companies, and Turkish and Russian bathing establishments. National and international associations¹ have been formed, to promote the movement. The increased interest taken in the sanitary condition of the pools is an indication of their growing popularity, and therefore of the practical importance of the subject.

That the swimming pool is actually a medium for the transmission of disease, has been well pointed out by Atkin² who divides diseases communicable from pool into three classes: (1), intestinal, (2) eye and ear, and (3) venereal. We follow his classification in the succeeding paragraphs.

1. *Intestinal infections*.—Jäger³ reported the occurrence of an intestinal proteus infection among ten soldiers in the garrison of Ulmer, who had bathed in the river Danube. Two of the patients died. The infection was traced to an epidemic of fowl disease in a village situated on a tributary of the Danube, a short distance above the place where the soldiers had bathed. Jäger found

* Received for publication March 7, 1914.

1. The American and The International Association for Promoting Hygiene and Public Baths.

2. *Proc. Ill. Water Supply Assoc.*, 1911, 3, p. 73.

3. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1892, 12, p. 525.

the bacilli in the water of this tributary, in the feces of the patients, and in the bodies of the dead fowls.

Drescher⁴ isolated a type of proteus bacillus from the water and from the feces of the patients during an epidemic among 38 soldiers who had bathed in the Neise. After the bathing in this river had been stopped the epidemic subsided.

Pfuhl⁵ attributed 49 cases of typhoid fever to bathing in the Elbe at Altona. Lenhartz⁶ gave an account of a case of typhoid fever in a man who had fallen into the Elbe and swallowed considerable water. Klein and Schütz⁷ reported cases of typhoid fever in 6 soldiers who had bathed in water close to the mouth of a city drainage canal. In the discussion of a paper by Maier,⁸ Dr. Reece reported the occurrence of 34 cases of enteric fever among soldiers who had bathed in a swimming pool which derived its water from a sewage-polluted river. About 10 per cent of the men using the pool became infected, while only one case developed among those who did not use the pool. The epidemic ceased when bathing in the pool was discontinued.

In Japan, Shiga investigated an epidemic of 413 cases of dysentery in the village of Mitaknura. Near the town was a river in which bathing had been prohibited. When this restriction was removed, hundreds of persons went swimming, and within four days the epidemic broke out. It was found that the clothes and bedding of a person, who had died of the disease, had been washed in the water of the stream a short distance above the village. Shiga concluded that the epidemic was due to the ingestion of the river water by bathers.

Shäfer,⁹ Witte,¹⁰ Kirchner,¹¹ Hartog,¹² Bassin,¹³ Baginsky,¹⁴ and others have further advanced presumptive evidence that intestinal diseases can result from bathing in polluted water.

2. *Eye and ear infections.*—Fehr¹⁵ tells of 20 cases of eye infection among patrons of a public swimming pool, and in one case reinfection occurred as a result of subsequent swimming in the same pool. Schultz¹⁶ reported 18 cases of trachoma among the young men who had used a public swimming pool which had been contaminated by an attendant who had sore eyes. Cobb¹⁷ reported two cases of ear infection from the use of a swimming pool. In one case the boy was infected three consecutive times from the same pool.

3. *Venereal diseases.*—Hertzka¹⁸ and Sticker¹⁹ have demonstrated that *B. prodigiosus* can pass into the vagina of women while swimming. Skutch²⁰ has reported an epidemic of gonorrheal vulvovaginitis which spread to 236 girls in a school at Posen. They had all used the same swimming pool, but not the

4. Sanitätsber. über d. kgl. Preuss. Armee, 1898.

5. Deutsche Mil. Wchnschr., 1888, 17, p. 9.

6. München. med. Wchnschr., 1892, 6, p. 898.

7. Wien. med. Wchnschr., 1898, 6, p. 238.

8. Proc. Roy. Med. and Chir. Soc., London, 1908, 2, p. 227.

9. Deutsche Mil. Wchnschr., 1890, 36, p. 39.

10. Sanitätsber. über d. kgl. Preuss. Armee, 1898.

11. Deutsche Mil. Wchnschr., 1888, 17, p. 95.

12. Sanitätsber. über d. kgl. Preuss. Armee, 1897.

13. Ibid., 1896.

14. Hyg. Rundschau, 1896, 6, p. 597.

15. Berl. klin. Wchnschr., 1900, 1, p. 37.

16. Ibid., 1899, 39, p. 36.

17. Boston Med. and Surg. Jour., 1908, 159, p. 9.

18. Monatschr. f. Geburtsh. u. Gynäk., 1902, 16, p. 3.

19. Ztschr. f. Geburtsh. u. Gynäk., 1901, 45, p. 510.

20. Centralbl. f. Bakteriöl., 1892, 12, p. 309.

same towels, soap, etc. Rosenau states: "Gonorrhea is usually transmitted by sexual congress; however, accidental or innocent infections are not infrequent." Paul Bending²¹ reports the case of 40 girls sent for convalescence to a brine bath, 15 showed signs of gonorrhea after the return. The infection came from an 8-year-old girl, who apparently had been suffering from gonorrhea for several years, and the disease was spread through indiscriminate bathing in one bath-tub and the use of the same bath towel.

That swimming pools can transmit disease, then, has been actually demonstrated. When, therefore, the use of the swimming pool is made compulsory, as it is, for example, in secondary schools in New York state, it would seem just that the authorities imposing this condition, be compelled to exercise sanitary supervision, and be held responsible for accidental infections traceable to defective sanitary management.

IMPORTANCE OF ANALYSIS FOR INTESTINAL BACTERIA

The final decision concerning the sanitary condition of a swimming pool must be based upon bacteriological examinations. The appearance of the water is practically of no significance, since it may be clouded by organic or inorganic matter in suspension. In our own investigations, pools that were dirty in appearance were often found to be relatively free from bacteria, and vice versa. Atkin²² and Manheimer²³ have pointed out that chemical analysis alone is of no value in determining the sanitary condition of the swimming pool water, since the large quantity of organic matter added by each swimmer makes the interpretation of the analysis difficult. Unlike the chemical analysis of drinking water, in which such analyses may be of considerable importance, it is worthless in the case of water of swimming pools. Bacteriological investigation therefore remains the only reliable index.

A review of the literature indicates that the most important diseases transmitted through the agency of the swimming pool are those which affect the intestinal canal. Typhoid fever and diarrheal conditions attributed to *B. proteus* and some other bacteria have been traced to swimming pool infection on reasonably reliable evidence. This being the case, the examination of the swimming pool water for colon bacilli as an index of pollution becomes as logical a method of control as it does in the control of drinking water.

Colon bacilli are usually absent in 1 c.c. of drinking water in and about New York City. This fact has been established by the repeated routine analyses of the Department of Water Supply, Gas, and Electricity, as well as by our own examinations, and, on the basis of such measurements, comparative examinations of the swimming pool waters may easily serve to determine the degree of pollution.

In New York City the question of typhoid fever assumes especial importance since the disease seems to be endemic. Jackson in his report to the

21. *München. med. Wchnschr.*, 1909, 56, p. 1864.

22. *Proc. Ill. Water Supply Assoc.*, 1911, 3, p. 73.

23. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

Merchants' Association of New York²⁴ attributes this to the fact that the tides are not sufficiently strong to carry away the solid masses of sewage, and, in consequence, masses of human excreta accumulate along the shores when the tide falls. In summer these are covered with flies and form a constant menace. The report of the Metropolitan Sewage Commission²⁵ also largely attributed the prevalence of typhoid fever to the vast quantity of sewage-polluted water that surrounds the city. Altho this condition may account for some of the cases, the pollution of swimming pools by typhoid carriers must be looked upon as a constant and important danger. That the typhoid carrier state is not as uncommon as was formerly supposed is evident from the numerous studies recently made. Since it would be hopeless for technical reasons (here, as well as in the case of drinking water) to rely upon the actual determination of typhoid bacilli themselves, the general index of pollution furnished by the colon test seems to offer the best means of controlling pollution. Unlike drinking water, in this case we can definitely exclude contamination from any but a human source. This exclusion increases the value of the examination, and standards of safety can be established.

Formerly, most of the endemic typhoid fever was attributed to the ingestion of polluted water. At the present time, it appears that the disease remains endemic largely because of the constant foci of infection supplied by temporary and permanent bacillus carriers. Russell²⁶ states: "In the southwestern part of Germany many bacillus carriers have been found, and the authorities have, at present, about three hundred of such persons under observation. Many, after a time, cease to be carriers, but new ones are continually being discovered, and so the number remains constantly high." Rosenau states that 4 per cent of all typhoid fever patients continue to discharge typhoid bacilli in the urine or feces, during and after convalescence. Albert states that 25 per cent of all chronic typhoid carriers have never had typhoid fever, and further estimates that 1 in every 1,000 of the general population is a carrier.

The very thorough studies of Rosenau, Lumsden and Kastle,²⁷ and of Lumsden and Anderson²⁸ on the rôle played by bacillus carriers in the spread of typhoid infection in the city of Washington, deserve especial attention. In 1908 these investigators examined the feces of a thousand different persons in apparently good health, or with maladies not suggesting typhoid fever. The results of their examinations indicated that 0.3 per cent of the general population of Washington became temporary typhoid bacillus carriers. In their search for chronic bacillus carriers, they examined 307 specimens of urine and feces of persons who had had the disease, and found that 8, or 2.8 per cent of these individuals were discharging typhoid bacilli. When one considers the difficulty of isolating typhoid bacilli from feces, and also the fact that carriers sometimes fail to discharge bacilli for considerable lengths of time, the estimation of the Washington investigators must be considered extremely conservative. In this connection it is well to mention Park's interesting case of "Typhoid Mary"²⁹ who infected twenty-six persons, some fatally, with typhoid fever. Bolduan and Noble³⁰ report the case of a dairyman who unwittingly dissemin-

24. *Merchants' Assoc. of N. Y.*, 1909.

25. *Rept. Metropol. Sewage Com. N. Y. City*, 1908.

26. *Mil. Surgeon*, 1909, 24, p. 53.

27. *Bull. Hyg. Lab., U. S. P. H. S.*, 1909, No. 53.

28. *Ibid.*, 1911, 78.

29. *Pathologic Bacteria and Protozoa*.

30. *Jour. Am. Med. Assn.*, 1912, 58, p. 7.

ated typhoid germs for forty-eight years. The literature of this subject has been fully reviewed by Ledingham³¹ and Grimm.³²

The connection of the foregoing considerations to the problem of the sanitation of the swimming pools is clear, and renders the determination of the degree of pollution by organisms that produce intestinal infections a matter of great importance.

LITERATURE AND FACTORS OF IMPORTANCE IN SWIMMING POOL SANITATION

The literature on swimming pools is not very extensive. Moreover, the points of view from which pool sanitation has been studied are so various that we can best survey the work that has been done by classifying it according to the particular features of this problem, with which each writer has concerned himself. The factors involved in the control of swimming pools consist of: (1) construction and equipment, (2) attention to the source of water, (3) management of the water, i.e., filtration and refiltration, etc., (4) chemical disinfection, and (5) administration. Judgment of the efficiency of any or all of these factors can be formed only by (6) bacteriological control which, therefore, we classify as a sixth important feature in sanitary management.

1. *Construction and equipment.*—Only brief mention of the structural features of swimming pools can be made in this paper. However, the details of construction are as essential to the sanitary efficiency as chemical treatment of the water or any other sanitary measure. Without entering at length into this subject we may summarize the chief sanitary features of construction and equipment of swimming pools as follows: the smoothness of the lining, lack of obstructions in the water, the presence of a combination of surface-overflows and life rails, adequate shower-bath and toilet facilities, and an efficient filtration plant.

2. *Attention to the source of water.*—It is perfectly obvious that the original water must be pure in a sanitary swimming pool. Manheimer³³ has shown that the water used in many out-of-town pools was of very poor quality. The summary of his results: number of pools using city and town supplies, 22; well water, 5; lake water, 4; river water, 2; creek water, 2. The lake, river and creek waters used, contained a considerable amount of sewage, while in two cases the State Department of Health had condemned the town supplies.

3. *Management of the water.*—*Refillings.*—Earlier writers, as Edel,³⁴ Hesse,³⁵ and others³⁶ have dwelt on the importance of frequent refillings. Manheimer³⁷ has more recently found that there is no direct relation between the number

31. *Rept. of Local Gov. Bd. of Eng. and Wales*, 1909, 38, p. 82.

32. *Pub. Health Repts.*, 1911, Washington, 26, p. 313.

33. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

34. *Arch. f. Hyg.*, 1893, 19, p. 225.

35. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1897, 25, p. 482.

36. *Hyg. Rundschau*, 1908, 18, 1391.

37. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

of weekly refillings and the sanitary condition of the water of a swimming pool.

Dilution.—Manheimer³⁸ has reported upon the effects of dilution in several pools. It appears that dilution of the water of a swimming pool is efficient, tho expensive.

Refiltration.—Schwartz³⁹ in 1905, was the first to investigate the refiltration of pool water. Kister and Fromme⁴⁰ highly advocated refiltration, and in addition to this, advised refillings every three weeks, with also the addition of considerable amounts of dilution water. Angel⁴¹ in a paper read in 1908, concluded that refiltration improved the appearance and bacteriological condition of the water, and also reduced the cost of maintenance. Crane⁴² and Manheimer⁴³ both recommended refiltration when combined with other methods.

4. *Chemical disinfection.*—Methods similar to those proposed for the purification of drinking water have been suggested for the chemical disinfection of the water of swimming pools. The first attempt in this direction was that of Stokes⁴⁴ who used copper sulphate to purify a Baltimore swimming tank and obtained destruction of bacteria and algae using 1 part of copper sulphate to 100,000 parts of water. Alexander⁴⁵ reported satisfactory results from the use of magnesium hypochlorite derived by electrolysis from a chemical containing magnesium chlorid. All other writers on this subject have recommended the use of calcium hypochlorite.⁴⁶ Burrage, in 1909 at Purdue University, made careful analyses of the bacterial condition of the water before and after disinfection with calcium hypochlorite. Following the recommendations formulated for the disinfection of drinking water by Woodhead⁴⁷ and others⁴⁸ he used one part of available chlorin⁴⁹ to one million parts of water. The counts, which averaged about 100,000 bacteria per c.c., were reduced by this means to between 0 and 26 per c.c. Bunker⁵⁰ in 1910, showed that with one part of available chlorin to a million parts of water, sterilization was effected in 15 minutes. Atkin⁵¹ in 1911, was successful when using 1 part of chlorin to

38. *Ibid.*

39. *Gesundts Ingenieur*, 1910, 30, p. 30.

40. *Ibid.*

41. *Assn. Mun. and County Eng.*, 1908, 34, p. 960.

42. *Eng. List*, 1912, 21, pp. 11, 43. *Proc. Am. Assn., Promote Hyg. and Pub. Baths*, 1913.

43. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

44. *Am. Med.*, 1905, 10, p. 1075.

45. *Scient. Am. Supp.*, 1909, 68, p. 1765.

46. The following are some of the cities in the United States and Canada, that use calcium hypochlorite for the purification of the drinking water: New York City, Jersey City, Council Bluffs, Brainerd, Erie, Montreal, Milwaukee, Cleveland, Ridgewood, Corning, Omaha, Nashville, Grand Rapids, Little Falls, Harrison, Baltimore, Cincinnati, Toronto, Niagara Falls, Minneapolis, Pittsburgh, Rahway, Hackensack, Ottumwa, etc. (Hooker).

47. *Jour. Roy. San. Inst.*, 1910, 31, p. 281.

48. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1895, 20, p. 227. *Lancet*, London, 1908, 2, p. 1597. *Pub. Health*, London, 1909, 23, p. 350. *Ztschr. j. Hyg. u. Infektionskrankh.*, 1894, 16, p. 149.

49. CaOCl_2 , calcium oxychlorid, is generally accepted to be the essential constituents of dry chlorid of lime. It is soluble in about twenty times its weight of water, leaving a small insoluble residue, mostly calcium hydroxid: In an aqueous solution, calcium hypochlorite forms the only valuable constituent, the calcium chlorid being inert and valueless. The reaction in water is $2\text{CaOCl}_2 = \text{CaCl}_2 + \text{Ca}(\text{OCl})_2$ (calcium hypochlorite).

From solution of hypochlorites, carbonic acid contained in the water will liberate free hypochlorous acid ($\text{Ca}(\text{OCl})_2 + \text{H}_2\text{CO}_3 = 2\text{HOCl} + \text{CaCO}_3$); the hypochlorous acid is the active oxidizing agent. It is clear from this, that the term "parts of available chlorin" is an expression used merely for convenience, and does not refer in any sense to the chemical reaction.

50. *Am. Jour. Publ. Health*, 1910, 20.

51. *Proc. Ill. Water Supply Assoc.*, 1911, 3, p. 73.

a 1,000,000 parts of water, but found that the effect of the hypochlorite was unsatisfactory when 0.5 part was used. Whipple,⁵² Lewis,⁵³ Rettger and Markley,⁵⁴ Lyster,⁵⁵ Tully,⁵⁶ *The Engineering Record* (unsigned),⁵⁷ Manheimer,⁵⁸ Ravenel,⁵⁹ Markley,⁶⁰ Bunker and Whipple,⁶¹ all have recommended the use of calcium hypochlorite for swimming pool disinfection on the basis of tests of its efficiency. More recently Buswell⁶² in 1913, following the work of Darnall,⁶³ suggests the use of anhydrous chlorin⁶⁴ as being more effective for bacterial destruction, easier of application in the pool he examined, and more constant in composition than calcium hypochlorite. He objects to the hypochlorite chiefly because of lack of constancy in composition.

5.—*Administration*.—Manheimer⁵⁸ has compared the sanitary condition of several pools that were operated in about the same manner, and had approximately the same cubic capacities. He found that the administration of the pool, the supervision of the working force, the inspection of the bathers before entering the water, and their instruction in pool sanitation were the most important agencies in keeping the bacterial counts low.

To complete the review of the literature of this subject, it is well to mention the papers of Roberts and Porter. Roberts,⁶⁰ besides urging the use of calcium hypochlorite, suggests the use of isotonic salt solution instead of common water for tanks, as the solution is less irritating to the mucuous membranes. He also suggests a circulating pump to insure the immediate distribution of added germs. Porter⁶⁷ confines himself in his paper to a careful consideration of the dangers associated with unsanitary pools, without considering recommendations for their control. A large part of his interesting communication relates to the "Kosher" baths used by the poorer class of Jewish people on the lower East Side of New York City. At his suggestion this investigation was taken up by us, and Porter's results will be discussed below.

PLAN OF STUDY

Our own plan of investigation consisted in making periodical bacteriological examinations of a considerable number of pools and Mikveh baths, attempting to find methods of operation that might be applied to the sanitary management of swimming pools in general. In addition to this, it has been our aim to correct individual procedures where these have been found faulty.

52. *Mun. Jour. and Engin.*, 1911, 31, p. 577.

53. *Eng. News*, 1911, 65, p. 636.

54. *Ibid.*, 66, p. 699.

55. *Jour. Am. Med. Assn.*, 1911, 57, p. 1992.

56. *Am. Jour. Pub. Health*, 1912, 11, p. 1186.

57. *Engineer Rec.*, 1912, 65, p. 699.

59. *Ibid.*, p. 684.

58. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

60. *Ibid.*, 1913.

61. *Ibid.*, p. 75.

62. *Ibid.*, p. 395.

63. *Jour. Am. Pub. Health Assn.*, 1911, 17, p. 78.

64. Anhydrous chlorin sterilizes by means of its nascent chlorin, whereas, chlorid of lime acts by means of the nascent oxygen liberated from hypochlorous acid. (See footnote above.)

65. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

66. *Eng. News*, 1912, 67, p. 73.

67. *Survey*, 1912, 28, p. 588.

TECHNIC OF BACTERIAL WATER ANALYSIS

Collection of samples.—Samples were collected from the surface, middle-depth, and bottom of the pools, except in cases mentioned in the text. The collection of these samples was always from the deep end of the pool. A weighted bottle (Abbott) was used for the collection of bottom and mid-depth samples, and a glass-stoppered one held by means of a bent wire for surface collections.

The tops of all bottles were wrapped in stiff brown paper, to protect the necks and lips from dust. The bottles were then sterilized in the hot air oven for one hour, at 150° C. The copper wire used to lower the weighted bottle was marked off in feet, therefore the mid-depth of the pool was easily found. When the bottom samples were being collected, the apparatus was lowered gently till it touched the floor of the pool, then raised a few inches, drawn to one side, and the stopper temporarily lifted.

During transportation of the samples, ice-packing was ordinarily not needed, as the temperature out-of-doors was sufficiently cold to preclude any considerable change in the samples. Furthermore, but one or two pools were examined at a time, and the samples reached the laboratory within 30 or 40 minutes after collection. In all cases when more than two sets of samples were collected on one trip they were packed in ice.

I. QUANTITATIVE ANALYSIS

Treatment of samples.—The samples were plated in agar and gelatin, counts for colon bacilli were made in Jackson's peptone-lactose-bile medium⁶⁸ and in thirteen instances qualitative analyses were made for the detection of typhoid bacilli. In the results of a few preliminary tests it was found that the proper dilution of pool water to be made prior to implantation was 1 to 10 and 1 in 100, using normal salt solution as a diluent. In the "Kosher" baths on the Lower East Side of New York City, dilutions were made in various degrees, from 1 in 10, up to 1 of pool water, to 10,000 of salt solution, as the pollution of these pools was found to be exceedingly high.

In order to have the error, which usually attends the reading of pipets, equal for all tests, dilutions were so made that 1 c.c. could always be used for implantation. Few dilution tubes were made at one time, so that the volume might not be reduced by evaporation. It was noticed that immediately after sterilization the contents of the tubes had diminished slightly, due, no doubt, to the expulsion of dissolved air. The bottoms of the menisci of these tubes were therefore marked (prior to sterilization) immediately after which they were placed in the ice-box until used. As a rule these tubes resumed their proper volume upon warming and shaking, but frequently several had to be discarded.

Plating.—When plating, 1 c.c. of the sample or its dilution was added directly to the plates, the standard agar or gelatin being poured over. The inversion of the agar plates was effective in preventing the water of condensation from running over the surface, and little or no trouble was experienced because of surface spreading. Some bacteria cannot withstand, even for a short time, temperatures above 45° C., therefore in order not to destroy any of these more delicate organisms, the agar and gelatin were cooled to between 40° and 45° C.

68. *Jour. Infect. Dis.*, 1910, 7, p. 587; *Ibid.*, 1909, 6, 289; *Ibid.*, 1911, 8, p. 289; *Jour. Ind. and Eng. Chem.*, 1909, 1, p. 328; *Jour. Infect. Dis.*, 1907, *Supp.*, 3, p. 300; *Ibid.*, 1911, 8, p. 241.

before plating. Duplicate plates were poured for each dilution, and several dilutions were made for each sample. When any wide discrepancy occurred between counts of different dilutions for the same sample, the test was discarded. In selecting the final count to be recorded in the table, those from the highest dilutions were used.

II. QUANTITATIVE ANALYSIS

The method used in the detection of colon bacilli in water, one of the most important parts of the analysis, varies but slightly from that used and described before.⁶⁹ Jackson's lactose-peptone-bile medium was prepared and placed in fermentation tubes, which were given fractional sterilization for 3 days in the autoclave at 15 pounds pressure, instead of 15 minutes as recommended. When these tubes are sterilized in the autoclave, their contents frequently boil up, wetting the cotton plugs, and perhaps some of the lactose is hydrolyzed by the extreme heat. Five tubes and sometimes 6 were used for each sample. To each of the first two, 1 c.c. of the pool water, and to each of the remaining three, 1 c.c. of a dilution, were added. Twenty per cent of gas in the closed arm of the fermentation tubes was considered positive for the presence of colon bacilli. Many tests were made to verify this fact,⁷⁰ but when there was the slightest doubt, the tubes were re-examined. In the analysis of the inhibition of growth due to temperatures about 37° C., it was found that very little, if any, inhibition occurred up to 42.5° C. Therefore a separate incubator was run at 42.5° C. and all colon bacilli and typhoid bacilli⁷¹ tests were made at that temperature. Inasmuch as each degree above 37° C. exerts additional inhibition to the growth of "water bacteria" it would seem that 42.5° C. is a better temperature than 37.5° C. for growth in all tubes for qualitative analysis.

CLASSIFICATION AND ARRANGEMENT OF MATERIAL

Of all the factors involved in the sanitary control of swimming pools, construction and equipment, source of water supply, etc., we have selected the factor of *administration* as the most convenient basis for classification. Accordingly we have divided pools into the following classes: (1) collegiate pools; (2) association pools; (3) public pools; (4) free floating baths; (5) Mikveh baths.

I. COLLEGIATE POOLS

The following three pools represent the most interesting and instructive group studied. It is in these pools that most of the experiments on technic and management have been worked out, and in their combined application an almost ideal procedure in the sanitary management of swimming pools is developed. The difficulties here

69. *Am. Phy. Ed. Rev.*, 1912, 17, p. 669.

70. *Ibid.*, 28, p. 588.

71. Careful and repeated examinations were made, by methods proved efficient in control, to isolate typhoid bacilli from the various pools. Since these were uniformly negative we will omit details.

encountered are far more amenable to control than those found in any other group.⁷²

POOL 1

Water:. Filtered city water. Capacity, 50,000 gallons. Total water used per week, 200,000 gallons. Cost for water, \$8.00 per week.

Management of water: Pressure filtration. Dilution: Pool half emptied each day. Entirely emptied twice a week, at which time thoroughly scrubbed.

Attendance: 800 per week, mainly college women, with a few elementary school boys.

BACTERIOLOGICAL EXAMINATION

	A. Quantitative Analysis		B. Qualitative Analysis for
	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	Colon Bacilli per c.c.
Tap water (control)	40	400	0
Water from pool before use.....	70	1,130	0
After 9 hours' use by 125 women	3,400	Not made	Bet. 1-10
After 30 hours' use by 250 women	12,500	Not made	Bet. 1-10
After dilution following morning..	9,540	45,790	0
Evening same day	6,000	Not made	Bet. 1-10

Discussion: Relatively poor results for amount of money expended in running pool.

POOL 2

Water: Filtered city water. Capacity, 100,000 gallons. Total water used per week, 450,000 gallons. Cost of water per week, \$14.00.

Management of water: Pressure filtration. Dilution: Constant stream of warm filtered water night and day, equivalent to four refillings per week. Pool thoroughly scrubbed once a week.

Attendance: 2,000 per week, mainly college men.

BACTERIOLOGICAL EXAMINATION

	A. Quantitative Analysis		B. Qualitative Analysis for
	Agar 37° C. per c.c.		Colon Bacilli per c.c.
After 1 day's use by 400 men.....	300		0
After 2 days' use by 800 men.....	1,000		Bet. 1-10
After 3 days' use by 1,200 men.....	3,000		Irregularly present
After 4 days' use by 1,600 men.....	16,500		Irregularly present

TEST OF EFFICIENCY OF FILTER

	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.
Tap water (control)	13	192 Water murky
1 day's attendance..... 0	0	3
2 days' attendance..... 0	20	10
3 days' attendance..... 0	2	35 Water murky
4 days' attendance..... 0	160	280

There were no colon bacilli present in 1 c.c. in any of the samples.

72. All bacteriological tests recorded throughout the following protocols are averages of three separate determinations.

Discussion: It was evident that the pool was in relatively good sanitary condition considering the large attendance. The filter, however, was worked beyond its capacity to clarify the water. It should be operated, therefore, more slowly. The cost of maintenance is unnecessarily high and can be reduced by methods described below.

POOL 3

Water: Filtered, refiltered city water. Capacity, 250,000 gallons. Water used for one or two months. Cost slight.

Management of water: Gravity sand filter, used for filtration and for refiltration. Anhydrous chlorin added in first series of tests, calcium hypochlorite in second. Pool filled once in two months, depending upon the results of appropriate tests.

BACTERIOLOGICAL EXAMINATION WITH ANHYDROUS CHLORIN

A. Quantitative Analysis

	Agar 37° C.	Gelatin 20° C.	B. Qualitative Analysis for Colon Bacilli
	per c.c.	per c.c.	per c.c.
Tap sample (control)	20	60	0
After 7 days' use by 1,800 men A. M.	5	540	0
After 11 days' use by 2,100 men P. M.	50	600	0
After 8 days' use by 2,100 men A. M.	50	290	0
After 11 days' use by 2,400 men P. M.	60	520	0
Test from filter.....	5	410	0

BACTERIOLOGICAL EXAMINATION WITH CALCIUM HYPOCHLORITE

A. Quantitative Analysis

	Agar 37° C.	B. Qualitative Analysis for Colon Bacilli
	per c.c.	per c.c.
After 7 days' use by 2,100 men.....	800	0
After 11 days' use by 3,300 men.....	2,300	0

Discussion: The water was in excellent condition throughout use. Anhydrous chlorin and calcium hypochlorite were equally efficient in reducing bacterial pollution. Refiltration kept water so clear that smallest object was discernible on the floor of the pool. Cost of maintenance greatly reduced. Technic of this pool is recommended.

SUMMARY

The three preceding pools have much in common: they are all collegiate pools accommodating an intelligent clientele, they are well directed, and all have instructors present to prevent accidental drowning, and to insure obedience to the rules of cleanliness. Pool 1 uses plenty of water, derived from nightly dilution and bi-weekly fillings. Pool 2 maintains constant dilution night and day. Pool 3 retains the same water for months, but refilters and disinfects with chlorid of lime. The procedure of Pool 3 is far superior, from the view points of appearance of the water, economy of control, and sanitary condition. This condition was obtained by chemical disinfection and refiltration. The bacterial condition of the water of this pool closely approximated that of drinking water, which, after all, is the standard that should be set for the sanitary condition of swimming pools.

II. ASSOCIATION POOLS

The association pools are managed as the collegiate pools and accommodate the same class of attendance. By charging admission to the association, they insure a higher and cleaner class of patrons than the public pools which are described later. The technic in operating the different association pools is widely dissimilar and therefore very instructive. Many pools were examined but those that had similar technic and sanitary conditions approximately the same, have been omitted. The pools have been subclassified according to the number of weekly fillings.

POOLS FILLED DAILY

POOL 4

Water: Artesian well, not filtered. Capacity, 15,500 gallons. Cost for water = cost for pumping and heating.

Management of water: Daily filling and cleaning.

Attendance: 75 men daily.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis	BACTERIOLOGICAL EXAMINATION		B. Qualitative Analysis for Colon Bacilli
	Agar 37° C.	Gelatin 20° C.	
	per c.c.	per c.c.	per c.c.
Test of well water.....	4	40	0
After day's use by 75 men.....	1,300	31,800	0

POOL 5

Water: Artesian well, not filtered. Capacity, 27,000 gallons. Cost of water = cost for pumping and heating.

Management of water: Daily filling and cleaning.

Attendance: 150 men daily.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis	BACTERIOLOGICAL EXAMINATION		B. Qualitative Analysis for Colon Bacilli
	Agar 37° C.	Gelatin 20° C.	
	per c.c.	per c.c.	per c.c.
Test of well water.....	8	60	0
After day's use by 150 men.....	200	4,100	0

Discussion: Daily filling is satisfactory, but the cost of operation for large pools is prohibitive. Furthermore, the bacteriological conditions of these two pools were not so good as Pool 3, which depended not on frequency of refilling, but on refiltration and chlorination.

POOL FILLED THREE TIMES A WEEK

POOL 6

Water: Artesian well, not filtered. Capacity, 35,000 gallons.

Management of water: Pool cleaned three times a week.

Attendance: 250 men per week.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis

B. Qualitative
Analysis for
Colon Bacilli
per c.c.

	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control)	10	750	0
After day's use by 100 men.....	1,500	75,000	Bet. 1- 10
A. M. of second day.....	69,960	154,540	Bet. 1- 10
After 2 days' use by 250 men.....	2,200	292,500	Bet. 10-100

Discussion: The water in this pool was considerably polluted, in spite of frequent fillings. Growth of bacteria occurred after the first day's use. The growth consisted mainly of saprophytes (gelatin count at room temperature) though some increase is indicated in the bacteria which grow at incubator temperature. The increase in number of colon bacilli was due to bathers and not to growth. The sanitary condition of this pool is poor and should be corrected by methods indicated elsewhere.

POOL FILLED TWICE A WEEK

POOL 7

Water: City, filtered, refiltered. Capacity, 36,000 gallons.

Management of water: Refiltration. No chemical added to water. Cleaned twice a week.

Attendance: 15 persons per week.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis

B. Qualitative
Analysis for
Colon Bacilli
per c.c.

	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control)	52	197	0
Filtered water from pool.....	26	254	0
After 9 hours' use by 5 men.....	3,000	13,000	0
After 33 hours' use by 10 men.....	1,000	12,000	0
After 57 hours' use by 15 men.....	7,000	7,000	0
Pool filled every 10 days.			
After 7 days' attendance.....	110	1,300	Bet. 1-10
After 8 days' attendance.....	470	1,300	0
After 9 days' attendance.....	800	280	Bet. 1-10

Discussion: The sanitary condition was fair, but could have been made entirely satisfactory by the addition of calcium hypochlorite. It is clear from these tests that refiltration alone is not satisfactory in reducing bacterial pollution, although it is effective in removing much of the suspended matter.

POOLS FILLED ONCE A WEEK

POOL 8

Water: City water, not filtered. Capacity, 24,600 gallons.

Management of water: Pool thoroughly diluted on the third day (Wednesday). Cleaned once a week.

Attendance: 450 men per week.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis

B. Qualitative
Analysis for
Colon Bacilli
per c.c.

	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control).....	150	190	0
After 1 day's use by 25 men.....	400	1,600	0
Before 3 days' use.....	24,440	11,440	0
After 3 days' use by 125 men.....	3,000	6,100	0
Before 6 days' use.....	200	7,600	0
After 6 days' use by 450 men.....	1,100	7,600	Bet. 1-10

Discussion: There was a gradual increase in pollution which was checked by the addition of the diluted water after the third day.

POOL 9

Water: City, filtered, refiltered. Capacity, 55,000 gallons.

Management of water: Constant refiltration. Constant dilution. Water from pool used for flushing toilets, urinals, etc., fresh water usually employed for this purpose added to pool instead. 145,000 gallons of water from pool used weekly. Hence pool practically filled two and one-half times per week.

Attendance: 1,300 men per week.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis

B. Qualitative
Analysis for
Colon Bacilli
per c.c.

	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control)	5	65	0
Pool water before use.....	43	42	0
After 12 hours' use by 250 men..	200	50,000	Irregular
A. M. 48 hours' use by 500 men..	120	360	0
P. M. 60 hours' use by 700 men..	700	5,000	Irregular
A. M. 98 hours' use by 1,300 men..	130	340	Irregular

Discussion: The good results obtained are due to refiltration and dilution. The use of a disinfectant, however, would eliminate the necessity for emptying the pool at the end of each week, and also improve the condition of the water.

POOL 10

Water: Double filtered city water. Capacity 32,500 gallons.

Management of water: Pool scrubbed once a week. Two feet of water drawn off daily and replaced with fresh water. Equivalent to about two fillings a week.

Attendance: 250 men per week.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis

B. Qualitative
Analysis for
Colon Bacilli
per c.c.

	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control)	11	480	0
Before use (from pool).....	4	38	0
Used 48 hours by 70 men.....	310	1,380	0
Used 60 hours by 110 men.....	1,300	20,000	Irregular
Used 108 hours by 250 men.....	1,000	90,000	Bet. 1-10

Discussion: Plumbing should be modified for refiltration. Water was kept comparatively clear by double filtration.

POOLS EMPTIED ONCE IN TWO WEEKS

POOL 11

Water: City, filtered. Capacity, 55,000 gallons.

Management of water: Dilution water added at night in a quantity sufficient to account for fillings every 3 days.

Attendance: 6,000 men in two weeks.

BACTERIOLOGICAL EXAMINATION

	A. Quantitative Analysis		B. Qualitative Analysis for Colon Bacilli per c.c.
	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control)	5	36	0
Used 1 hour by 20 men.....	30	300	0
Used 12 hours by 500 men.....	700	6,000	Bet. 1-10
A. M. 96 hours by 1,000 men.....	4,000	44,000	Bet. 1-10
P. M. 108 hours by 1,500 men.....	2,500	150,000	Bet. 1-10
A. M. 210 hours by 3,500 men.....	2,600	150,000	Bet. 1-10
P. M. 222 hours by 4,000 men.....	5,000	170,000	Bet. 1-10
P. M. 300 hours by 6,000 men.....	1,400	31,800	0
Sample from filter (control).....	8	40	0

Discussion: It is obvious that sanitary measures are necessary to correct the needlessly high bacterial counts.

POOL 12

Water: City, filtered. Capacity, 90,000 gallons.

Management of water: Pool well filtered, clear throughout use. Can be arranged for three depths. Turkish baths (usually) taken prior to use of pool.

Attendance: 530 men in two weeks.

BACTERIOLOGICAL EXAMINATION

	A. Quantitative Analysis		B. Qualitative Analysis for Colon Bacilli per c.c.
	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	
Tap water (control)	5	254	0
After 168 hours' use by 180 men...	150	350	0
After 180 hours' use by 200 men...	1,000	10,000	Bet. 1-10
After 360 hours' use by 450 men...	200	6,000	0
After 372 hours' use by 480 men...	200	1,000	0
After 384 hours' use by 480 men...	180	230	0
After 396 hours' use by 500 men...	300	3,000	0
After 408 hours' use by 500 men...	180	300	Bet. 1-10
After 420 hours' use by 530 men...	500	2,000	Bet. 1-10

Discussion: The reasons for this sanitary condition are: (1) The body of water is large as compared with the attendance; (2) enough dilution water is added to account for a little less than one refilling; (3) most of the bathers had taken Turkish baths prior to entering the water.

POOL 13

Water: City filtered, refiltered. Capacity, 33,000 gallons.

Management of water: Water was kept clear by refiltration. Pool scrubbed every 2 weeks.

Attendance: Variable; 700 attended during period of examination.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis			B. Qualitative Analysis for Colon Bacilli
	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	per c.c.
Tap water (control).....	150	690	0
After 72 hours' use by 350 men...	200	25,000	0
After 108 hours' use by 700 men...	6,000	44,000	Bet. 1-10

SUMMARY

Pools that were filled once in two weeks showed varying sanitary conditions, depending upon the quality of the attendance, the amount of water offered per capita, the amount of dilution water, and the preliminary baths taken by the patrons. Undoubtedly the use of chlorid of lime, and of refiltration would improve these plants. The adoption of this technic is urged.

III. POOLS OPEN TO PUBLIC

Pools Nos. 14, 15 and 16 are open to the general public with no admission fee and no restriction, except good behavior. They are attended generally by a fairly good class of people. In the afternoons, mainly high school boys or girls bathe there; in the evenings, men or women. No instruction at public expense is attempted; the authorities merely open them and try to keep them clean. Men and women use these pools on alternate days. Pool 17, located in a high school, is also open to the general public in the evenings. During the day, however, it is maintained as a part of the regular instruction in physical training for the pupils.

POOL 14

Water: City, filtered. Capacity, 83,000 gallons. Filled every 2 days.

Management of water: Scrubbed four times a week. Women use the pool when first filled because of the ease with which gonorrhea may be contracted by women in polluted water. Cost of maintenance⁷³ \$29,789.49 per annum, exclusive of the cost of water. Bathers must be clothed.

Attendance: 200 women, 1,600 men, daily.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis			B. Qualitative Analysis for Colon Bacilli
	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	per c.c.
Tap water (control)	12	137	0
After use by 202 women.....	2,500	15,000	Bet. 1-100
After use by 1,600 men.....	400,000	600,000	Bet. 10-100

73. Rept. Borough Pres. of Manhattan on Pub. Baths, New York City, 1912.

POOL 15

Water: City, filtered. Capacity, 75,000 gallons. Filled three times a week.

Management of water: Women use pool when first filled. Pool scrubbed three times a week. Cost of operation, exclusive of cost of water, \$29,170.82. Patrons must wear apparel in water.

Attendance: Women, 300 daily; men, 600 daily.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis			B. Qualitative
	Agar 37° C.	Gelatin 20° C.	Analysis for Colon Bacilli
	per c.c.	per c.c.	per c.c.
Tap water (control)	10	280	0
Filtered water	9	300	0
Used 1 hour by 20 women	50	7,000	Irregular
Used 15 hours by 300 women	3,000	50,000	Bet. 1-10
Used 38 hours by 600 men	20,000	60,000	Bet. 1-10

POOL 16

Water: City, unfiltered. Capacity, 90,000 gallons. Refilled three times per week.

Management of water: Same as previous pool, and in addition a "large handful" of chlorid of lime is added to water. The amount added is insufficient for disinfection. Wearing of apparel in water discouraged, though permitted.

Attendance: 200 women and 500 men daily.

BACTERIOLOGICAL EXAMINATION

A. Quantitative Analysis			B. Qualitative
	Agar 37° C.	Gelatin 20° C.	Analysis for Colon Bacilli
	per c.c.	per c.c.	per c.c.
Tap water (control)	20	80	0
After 13 hours' use by 200 women	25,000	31,000	0
After 48 hours' use by 200 women plus 500 men.....	85,000	160,000	Bet. 1-10

The practice of wearing clothing in the water is discouraged here, but insisted upon in Pools 14, 15. It would seem desirable for the practice in public pools to become standard. If clothing must be worn, it should be supplied by the authorities, with cleanliness guaranteed. The apparel worn by bathers frequently dyes the water. The expense of furnishing apparel could be eliminated by naked bathing for men. All the association, high-school, and collegiate pools maintain this practice with no noticeable ill effects.

The three pools discussed above (Nos. 14, 15 and 16,) are very similar in equipment, operation and sanitary condition. They fall into the same category because they are maintained at public expense, and their use is free of charge. No satisfactory methods for safeguarding the health of the patrons are employed and the use of chlorid of lime has been suggested, with the hope that this will effectively solve the problem.

These baths have been established to encourage swimming, as the death rate from accidental drowning is very high. Along the shores in fine weather, hundreds of persons, many nude, bathe in sewer-polluted water, risking personal health and safety. To offset this, the authorities established the free floating baths described later and the indoor plunges. The purpose of these baths and plunges, in addition to correcting the unsightly appearance of promiscuous and unlawful bathing from the docks, was to teach swimming to those anxious to learn. No instruction in swimming is given, however, and the very laudable purpose for which they were established has been defeated.

To correct this condition, a complete change in the administrative policy of these pools would be necessary. Our recommendations in this regard are: Instead of allowing the promiscuous use of these baths to any one, registration should be required, and a fee charged. At the time of application, the prospective pupil should be subjected to an examination by a physician of the Department of Health. If registration times were properly arranged in a season of the year when the physicians assigned by the Board of Health to examine school children were not busy, as for example during July, August and the beginning of September, and also several weeks throughout the year, these examinations could be given without incurring any additional expense. A set of rules should be given each applicant, covering the important items of sanitary conduct in the water, e. g., the importance of showering before and after bathing, and the importance of taking a sitz shower with soap (see summary), the danger from expectorating into the water, the importance of emptying the bladder before entering the pool, etc. Violation of these rules should result in expulsion, and forfeiture of the registration fee.

Each pupil should be assigned a convenient hour, and instruction in swimming should then be given. If, and upon good attendance, the pupil has learned to swim, his registration fee should be returned. On the other hand, if he attends irregularly, or fails to learn to swim after a stated length of time, there should be additional registration, and an additional fee charged. Separate hours should be set aside for all persons who can swim, registration should be required, but no fee charged. It is felt that at least two important purposes would be accomplished by this plan: (1) persons would be taught to swim, and others would be encouraged to attend who otherwise would never think of doing so; (2) some restriction on the admission of diseased persons to the pool would result. Many persons who are now afraid to enter the water, for fear of contamination, would be encouraged to do so by this plan.

POOL 17

This pool presents a problem unique in itself, and therefore needs separate treatment. It is patronized during the day by high school boys, being an integral part of their instruction in physical training; during the evening by the male public, over 16 years of age, without any restriction in, or any charge for its use.

Water: City, not filtered. Capacity, 24,500 gallons.

Management of water: Chlorid of lime added to water.¹

1. (This pool was examined at a time when chlorid of lime was not added. The bacterial counts ranged above 100,000 per c.c. Between 10-100 colon bacilli per c.c. were usually present).

BACTERIOLOGICAL EXAMINATION

	A. Quantitative Analysis		B. Qualitative Analysis for Colon Bacilli	
	Agar 37° C. per c.c.	Gelatin 20° C. per c.c.	Analysis for Colon Bacilli per c.c.	
Tap water (control).....	12	35	0	
After 12 hours' use by 400 persons	1,000	20,000	Bet. 1-10	
After 48 hours' use by 800 persons	5,000	75,000	Bet. 1-10	
After 72 hours' use by 1,200 persons	800	35,000	Bet. 1-10	

Discussion: This pool, even with the hypochlorite treatment, was not in satisfactory condition; the counts tho ordinarily not high, reached 163,360 per c.c. The technic of adding chlorin was poor and should be modified till a proper sanitary condition is obtained. The proper times to add the chlorid of lime are the following: In the evenings, after use by the general public, during lunch time, when no one uses the pool, and at 4 p. m. when the high school students are dismissed. The pool is not used between 4:00 p. m. and 7:30 p. m., thus affording an excellent opportunity for chemical treatment. The treatment should be controlled by appropriate tests, as described below, and would result in a much better condition of the water.

The use of the pool in the evening by the general public is subject to the same objections as described for pools Nos. 14, 15 and 16. No instruction in swimming is given, and no supervision of any kind to safeguard the health of the bathers is maintained. The attendance in the evening at the time of the examination did not exceed thirty, a number that could easily have been accommodated by Pool 14, which is in the vicinity. Radical change is here necessary to give a fair return to the city for the money expended, and to safeguard the health of high school boys. If this pool is to be continued and used, certain changes in administration and equipment are necessary. The use of the pool in the evening should be organized somewhat after the plan suggested for the previous three. Then again the pool should be deepened, as it is now so shallow that diving is dangerous. This could be done by building up the sides and utilizing the reconstruction for adding a combination life-rail and scum gutter, which is now absent. This would increase the cubic capacity of the pool, now insufficient. A filtering plant should be installed and so arranged that the water could be recirculated and refiltered in about six hours. The raising of the sides would not be difficult, and a ledge could be made sufficiently wide to permit the swimmers to stand upon it without danger. These recommendations are somewhat costly, but if carried out would rehabilitate an otherwise inadequate plant.

IV. OUTDOOR FLOATING BATHS OF THE CITY OF NEW YORK

The city of New York operates about 15 free floating baths, stationed during the summer at docks as far away from sewer outlets as possible. They are 95 feet long, and 60 feet wide, and are floated on 8 pontoons, four on each side. The peripheral parts of the pool and a balcony above are used for dressing rooms. The pool itself is divided into two parts, one 2.5 feet deep for children, and the other 4.5 feet deep for the adults. The average cost of construction of these

baths, twenty years ago, was about \$12,000, and their cost of maintenance for the three months of their use averages \$3,848.56.⁷⁴

Observations as to their sanitary condition have been repeatedly made by the writer since 1910. Bacteriological tests were made in 1911, but since that time the authorities have become aware of the polluted condition of the baths, and it is hoped that in the near future some provision for their purification will be made.

The bacterial counts on gelatin ranged between 500,000-1,000,000 per c.c.; 100 colon bacilli per c.c. were always obtainable and the presence of free floating feces was frequently observed. Other debris from the sewers and from the many passenger steamers that pass up and down the Hudson and East Rivers can be found in these baths, but obvious pollution does not interfere with the patronage of the general public. The Metropolitan Sewage Commission⁷⁵ has pointed out clearly their unsanitary condition and recommends their abolition, but has not, unfortunately, made any recommendation for substitutes. "Bathing in free floating bathing establishments may be refreshing, and may give pleasure to the bathers, some of whom thus learn to swim, but the baths have little cleansing value. The water is unwholesome and even dangerous for bathing purposes. Floating particles of sewage enter many of the pools, even when situated 500 feet or more from a sewer outlet. When bathing, it is hardly possible to avoid accidentally taking some of the water into the mouth and nasal passages, and having it come in contact with the mucuous membrane of the eye. It is not to be doubted that bathing in such waters is a frequent source of infection. . . . As soon as possible the maintenance of free floating bath establishments should be discontinued."⁷⁶

At the Baltimore convention on public baths when New York's floating baths were discussed, their remodeling into baths, using city (Croton) water, was urged. The cost of making the tanks water-tight and using water from the city main would be very great, and considering that such bathing establishments can be used only a few months in the year this expense seems unwarranted. The only excuse for floating baths, in place of the ultimately more economical indoor pools, is the prohibitive cost of land in the crowded districts of New York, where such baths are needed. Floating baths, if they must be used, should be made water tight, and the water from the river pumped in and filtered. In conjunction with the filtration, which should be effective in removing suspended matter, bleaching powder should be added to the water in an amount controlled by tests and sufficient to destroy all bacteria not retained by the filters.

Inasmuch as such baths are available only for a short time of the year, and as the expense of their remodeling would be great, it would seem that the establishment of public indoor pools on the end of the recreation piers would be ultimately more economical. It is the present policy of New York to increase its recreation facilities, and the erection of more public piers, with pools at one end, would not be difficult. These pools could form a regular part of the recreation equipment, and water from the river could be pumped into them, filtered, purified by chlorid of lime, and heated during winter, so that their use might become permanent. River water⁷⁷ should be used, largely because

74. *Rept. of Borough President of Manhattan on Public Baths, New York City, 1912, p. 10.*

75. *Rept. of Metropolitan Sewage Com., New York City, 1908-10.*

76. *Rept. of Metropolitan Sewage Com., New York City, 1910, p. 82.*

77. With the opening of the new Catskill aqueduct, New York City will be supplied with a superabundance of water. It will be cheaper and better at that time to use city water and add sea-salt to any desired concentration. Prof. W. H. Park suggested this and also that the cost of sea-salt would be far less than that of chlorid of lime.

of economy. Some writers, however, believe that salt baths are preferred by the people, which fact, together with the previous one, forms sufficient arguments for the use of river versus Croton water.

V. MIKVEH PLUNGES OF THE LOWER EAST SIDE OF NEW YORK

The attention of the writer was directed to these pools by H. F. J. Porter, M. E. industrial engineer, who believed that additional investigation and publicity would be productive of much good. All the data collected and used by him in the preparation of a comprehensive paper on this subject were turned over to the writer and used as a comparison and control with the data reported in the table below.

These pools are patronized by the Jewish people of the lower East Side of New York City. At certain times in the year (e. g. the Pass-over) their use is compelled for both men and women. Religious law, for example, forces the women to use these pools within seven days after menstruation. The Hebrew law⁷⁸ is very strict, stating that after thorough cleansing, the person should immerse himself or herself in a purified plunge filled with uncontaminated water, i. e., rain water, ice-water, or water that had not been polluted by human beings, and that this plunge should contain at least three cubic yards of water.⁷⁹ These excellent laws are not carried out. The men and women in their respective pools wash themselves in polluted water, in plunges with about 200 cubic feet capacities, and which are sometimes used by 300 different individuals without change of water. In connection with some of these Mikvehs there are either tubs or shower-baths, but neither of these is frequently patronized because of an additional charge of five cents for use. Hence the same water through constant use becomes contaminated and in no sense fulfils the requirements of the Biblical law.

Number of Mikvehs having between			
100-	1,000 bacteria per c.c.....	2*	
1,000-	10,000 bacteria per c.c.....	7	
10,000-	50,000 bacteria per c.c.....	4	
50,000-	100,000 bacteria per c.c.....	3	
100,000-	200,000 bacteria per c.c.....	7	
200,000-	500,000 bacteria per c.c.....	3	
500,000-	1,000,000 bacteria per c.c.....	4	
1,000,000-	or over bacteria per c.c.....	11	
Total plunges examined			41

78. *Bk. 3, Ch. 1, p. 11 of Levit.*

79. Biblical data furnished me through the courtesy of Rabbi Ch. Hirschensohn of Hoboken, New Jersey.

Number of Mikvehs having between		
0-	0 colon per c.c.....	3*
0-	10 colon per c.c.....	7
10-	100 colon per c.c.....	6
100-	1,000 colon per c.c.....	13
1,000-	10,000 colon per c.c.....	11
10,000-or more	colon bacilli per c.c.....	1
Total		41

* Attendance less than 3.

Forty-one of these baths were examined in 23 different buildings.⁸⁰ There are usually, in one building, several plunges containing water at different temperatures. The bathers go from the lower to the higher temperatures where they remain for several minutes till they have sweated thoroughly, thus removing most of the dirt. When sweating-rooms are provided, bathers often prefer to use them in place of the Mikvehs. The temperatures of these plunges range from 18°-50° C. After use the water is thick, slimy, and unpleasantly odorous. The average attendance varies, in several instances 80-100 persons bathed in 200 cubic feet of water, while in one instance 300 persons used a slightly larger pool.

There can be small wonder, therefore, at the very large bacterial findings. Counts on gelatin ranged from 1,000 to 3,000,000 bacteria per c.c. and counts on agar were as high, in fact, in one instance they reached 18,144,000 bacteria per c.c. The counts for colon bacilli are interesting and astonishing. In the instance where 300 persons had used the water the colon count was 10,000 per c.c. The temperature of this pool (50° C.) was sufficiently high to continuously destroy colon bacilli. The large number were present in spite of this fact.

That these pools are a serious menace to the health of the people using them is clear. Radical measures to improve their condition or to substitute other means of ablution should be immediate. This neighborhood on the lower East Side is extremely crowded, and the tenements are not equipped with bath-tubs. The people of these districts, therefore, must depend either upon the very few public baths established in these neighborhoods or these unsanitary Mikvehs.

In spite of the fact that these Mikvehs do not comply with the Mosaic Laws, their abolition would be strongly opposed by the Jewish people who bathe there. The writer believes that the following recommendations could be made which would insure cleanliness and at the same time permit the exercise of the required religious ritual:

1. The basements of public schools could be modified into public shower-bath rooms. The authorities of many foreign cities finding this method successful have encouraged the use of these baths not only as a regular part of the elementary school instruction, but also as a means of bathing for the general public. Such baths have been established in several public schools in New York City, but the use of these has been restricted to students. Shower-baths for adults would be in beneficial competition with the Mikvehs, especially if the baths were free of charge. That this plan is feasible and economical is unquestionable. It is preferable to the erection of new buildings because of the high

80. An inspector of the Department of Health of the City of New York was assigned by courtesy of the Commissioner to accompany us. Mr. Kranz acted as interpreter.

cost of land, the cost of erection, and the duplication of running expenses, such as the salaries for janitors, cleaners, etc. The basements of public schools are at present frequently used as playgrounds for which purpose the roof would be far superior. The use of the basements or parts of the basements, in the evenings, for shower-bath rooms would be a great benefit to the people of these crowded districts and would reduce the cost of public bath.

2. Jewish charities should be encouraged to establish sanitary bathing places free of charge, either in connection with Synagogues or elsewhere.

3. Settlements in the vicinity should make an effort to install showers for the adults of the neighborhood.

4. The authorities of New York City should pass sanitary regulations with which the proprietors of these establishments should comply in order to have their licenses renewed. Because a fee is charged these baths are licensed by the city, and through this licensing power the city can compel the enforcement of sanitary laws. Such laws should include the following requirements:

A. All Mikveh plunges should be abolished, because of their unsanitary and polluted condition. Mikvehs consisting of individual tubs might be substituted in their place.

B. Shower-bath establishments, with or without sweat-rooms, because of their cleaner nature, should be approved. The reduction of their license fee might encourage their increase.

C. All individual tubs should be cleansed after each bath. Failure to observe this precaution should cause forfeiture of license.

SUMMARY AND CONCLUSIONS

The literature on swimming pools shows that a number of maladies—infections of the eye and ear, gonorrhea, and intestinal diseases—may be transmitted through the water in the pools. Since typhoid fever is endemic in New York City, the danger of a typhoid carrier contaminating a pool is not remote. Therefore an accurate determination of contamination with intestinal bacteria is one of the most important indices to the sanitary condition of a swimming pool.

The present investigation has included examinations of five distinct types of swimming pools, all of which will be briefly discussed below.

I. Collegiate pools in the city of New York are in very good condition, owing to the careful supervision of the plants by the professors in charge, the knowledge of sanitation of the swimming pool possessed by its patrons, and finally because of the enforcement of the sanitary regulations by swimming teachers.

II. Association pools were divided into groups according to the number of weekly refillings. Those that were emptied daily were in good condition. The value of daily filling, however, is inferior, from a sanitary point of view, to refiltration and chlorination, and from an economic point of view, too costly. The pool emptied three times a week was in poor sanitary condition on the second day of use.

Chlorin should be added daily. The installation of a filter at a cost of \$500 would be an eventual economy.

In the pool emptied twice a week with refiltration practiced the use of a disinfectant was necessary. Refiltration unaided by chlorination is not a satisfactory method for the sanitation of swimming pools.

Among the pools emptied once a week, Nos. 8 and 10 needed refiltration and chlorination. Pool 9, in which refiltration was practiced and considerable dilution water added, was located on the top floor of the building. The water used to flush toilets and urinals and for all other waste purposes was subtracted from the pool and fresh water added in its place. This plan deserves imitation. The use of small amounts of chlorin here would put the finishing touches on an almost ideal pool.

In the three pools that were emptied every two weeks, Pool 11 could have its plumbing modified and refiltration installed at an expense of \$70; the excellent condition of Pool 12 was due to the fact that a large amount of water was used for a relatively small attendance and also members always took a careful preliminary bath and frequently Turkish baths; Pool 13, though maintaining a clear water by refiltration, required disinfection, an additional proof that refiltration unaided is not a sufficient means of maintaining a sanitary condition of the water.

III. Public pools in New York city are elaborately equipped and generously operated, but because of their large and promiscuous attendance, faulty technic and improper organization, they are possible sources for the spread of disease.

A much better condition of the water could be obtained by the use of chlorid of lime, the addition of considerable dilution water, and frequent refillings, or by the combination of chlorination and refiltration.

These plants would be of greater service if they were reorganized, all prospective pupils registered and examined by physicians of the department of health, diseased persons barred from admission, and instruction in swimming given.

IV. An examination of free floating baths in New York city showed the sanitary condition to be so poor as to warrant their abolition or immediate remodeling. If remodeled, they should be enclosed, made water-tight, and the river water pumped through a filter into them.⁸¹

81. See foot-note 76.

Inasmuch as filtration would not sufficiently reduce the bacterial pollution, the water should be subsequently disinfected with chlorid of lime. These floating baths can be used only a few months in the year, are, comparatively speaking, inaccessible, and are inferior in every way to the more attractive indoor plunges which can be used the whole year. The only excuse for these establishments is the saving in the cost of land, which is excessive in the crowded districts where they are most needed. The cost of constructing new indoor pools on the ends of the piers would be no more than the cost of remodeling the old scows into water-tight boats. This plan of establishing pools at the end of the recreation piers would be feasible and economical because:

1. The policy of the city in pursuing its plan of recreation, is to increase the number of these piers, which are already numerous.
2. There would be no extra cost of land, no extra cost of janitorial fees, and the cost of remodeling old scows would be as great, if not greater, than the cost of building model plunges on land.
3. The river water in which the people delight to bathe could be pumped in through a filter and subsequently disinfected.
4. The instruction in swimming could be correlated with the plan of recreation, and this instruction maintained the year round.
5. These pools would cost less to operate than the present public plunges. Water could be used more lavishly, and the pools operated throughout the entire year.
6. The cost of berths (docking fee) for floating baths would be saved.

V. Mikveh plunges in New York city are not sanitary and bathing in them menaces the health of the people. Jewish people in this vicinity wish them both for religion and for cleanliness, but the manner in which they are operated defeats these purposes. Individual tubs or Mikvehs to be cleansed after each bath, should be constructed in place of plunge Mikvehs. Licenses should be granted by the city, only after inspection shows entire compliance with any new sanitary laws

Inasmuch as the tenements of this vicinity have no bathing facilities, the basements of public schools should be remodeled into shower-bath rooms. Settlements in the district should open shower rooms to accommodate the adult population of the neighborhood, and Jewish charities should be urged to provide adequate accommodations for the

baths of their people. Synagogues should build Mikvehs that comply with the sanitary intent of the old Mosaic laws.

In view of the large number of plunges in New York city, and of their various sanitary conditions, an officer of the department of health should be appointed to supervise them. He should examine their sanitary conditions, grant licenses and approve or modify their methods of operation.

So far we have made many recommendations which we believe would tend to improve the conditions found in individual plants. We wish to summarize finally, general recommendations which we feel certain should be observed in all pools whatever their location or administration.

GENERAL RECOMMENDATIONS

1. *Construction and equipment.*—Pools should be constructed of smooth lining without crevices and should be of as large capacity as possible. There should be no obstructions of any kind in the water, a combination of life-rail and overflow ledge making this possible. Where feasible, the plunge should be constructed on the top floor of the building so that the water used for flushing purposes could be taken from it. The fresh water usually employed for this purpose should be added to the pool instead. Each establishment should be provided with adequate shower baths and convenient dressing rooms and toilets.

2. *Source of water supply.*—The water used in swimming pools should be pure. Where this is not possible it should be thoroughly purified before use by the methods indicated.

3. *Management of the water.*—The water should be thoroughly filtered before passing into the tank, so that the opacity of the water could not obscure a submerged person. The water should be changed frequently, and as much dilution water added as possible. These two procedures in themselves, however, are of little importance if refiltration and chemical disinfection are used.

Refiltration is an efficient and economical method of keeping water clear during protracted use.

4. *Chemical disinfection.*—Calcium hypochlorite, used in amounts controlled by appropriate tests, has been shown to be efficient for the disinfection of swimming pool water, and its application to the water, in conjunction with refiltration is urged as a most effective method of

pool sanitation. There are two simple ways of adding the chemical to the water: (1) Small cheese-cloth bags containing the hypochlorite may be strung along a pole of sufficient length to reach across the pool, and then dragged back and forth till the contents are dissolved. At the end of a half hour, the amount of chlorin remaining in the water should be measured (as previously described) by the man in charge. In the event of too much chlorin being added, the water of the pool might be diluted by sending in a stream of fresh warm filtered water. If too little hypochlorite has been added, the process of treatment should be repeated. (2) If the pool is to be subjected to refiltration the above procedure could be modified and simplified; instead of treating the pool by means of cheese-cloth bags, a small mixing and feeding chamber could be made and attached to the intake pipe, and the water after refiltration, continuously treated with hypochlorite. The question⁸² arises whether or not the slow gradual addition of the chemical is as efficient as is the rapid periodic method. With the slow method, the concentration of the hypochlorite is never great, while with the rapid, the sudden increase of the chemical concentration is sufficient to cause rapid bacterial destruction. When the chemical is added slowly, however, the time of its contact with the bacteria is accordingly prolonged, and this explains the equality in the efficiency of both methods.

Comparing pools where one or the other method is employed we find that the slow gradual addition of the chemical seems to be superior. This latter method also, however, should be controlled by tests. The attendant in charge, therefore, should take samples for examination; the amount of chlorin in the water will indicate to him its approximate purity, and should there be any unusual deviation from the reaction described below, it can easily be corrected. This method of using refiltration and chlorination appears to the writer to be the better, for the following reasons: (1) the clarity of the water throughout use would be maintained, and its sanitary condition improved; (2) the water could be used for several weeks, with an elimination of the necessity for cleansing the floor and sides of the pool, at the same time cutting down the great waste of water, and the amount of coal used for heating purposes.

A simple method for testing the amount of chlorin in water is as follows: To a liter of water in a flask, held over a white tile, should

82. This question was suggested to Professor Park.

be added a mixture containing a crystal of iodid of potassium, a few drops of acetic acid, and a teaspoonful of starch.⁸³ The proper end reaction to be obtained is a violet blue; if a darker color is obtained, too much chlorin is present, if a lighter, not enough is present.⁸⁴

After emptying the pool, its floors and sides should be washed with antiseptics (chlorid of lime, formalin, etc.), as pools not employing this precaution are frequently polluted prior to use.

5. *Administration.*—Students and patrons should be subjected to physical examinations before admission to the plunge, and all diseased persons excluded. A set of rules should be given covering the important items of sanitary conduct in the water, e.g., the importance of showering before and after bathing, the importance of taking sitz-showers⁸⁵ with soap, of abstaining from expectorating into the water, the importance of emptying the bladder before entering the pool, etc. Patrons and students should be compelled either to bathe nude, or to use clothing the cleanliness of which has been approved by the director.

In a plant properly managed the filters during use should be frequently reversed, thus washing the accumulated dirt into the sewer. When filling the pool, reversing every hour is usually found necessary. When refiltering the water from the pool, the reversing at least twice a day is necessary. The workmen in charge of the filtering plant and those employed to clean the pool, should be carefully supervised.

The writer wishes to express his gratitude to both Dr. Hans Zinsser and Dr. Augustus B. Wadsworth, for the material aid given him in the preparation of this paper.

83. The iodo-starch reaction is sufficiently delicate to indicate the presence of one part of chlorin in ten million parts of water.

84. *Jour. Roy. San. Inst.*, 1910, 31, p. 281.

85. The showers taken by swimmers, prior to entering a pool, are hardly sufficient to wash from their bodies the harmless saprophytes that are on their skin, and some method of washing the perineal regions should be employed. A simple perforated ring through which water could be made to flow or which would cause water to flow automatically when pressure is exerted on the ring might be satisfactory. As far as the writer knows, no arrangement of this kind has been devised.

STUDIES ON THE CLASSIFICATION OF THE COLON GROUP *

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The first biologic division of the colon group was made by Smith¹ in 1895. He divided all lactose-fermenting, non-liquefying organisms into groups according to their fermentation of saccharose. This classification was corroborated by many other workers and has found general acceptance. In 1905 MacConkey² suggested a further subdivision of these two groups based on the power of the organisms to ferment dulcitate. Not satisfied that this classification distinguished sufficiently well the different types of organisms belonging to the colon groups, he later³ proposed a further subdivision based on a number of biochemical differences, such as fermentation of certain other sugars, indol production, Voges Proskauer reaction, etc. Jackson,⁴ following MacConkey's lead, separated the group into sixteen distinct types.

The first attempt at a biologic classification based on the biometric principles, as outlined by the Winslows in 1908,⁵ was made by Howe in 1912.⁶ He worked with 540 strains of lactose-fermenting, non-liquefying bacteria and concluded that motility, quantity of gas, fermentation of mannite, dulcitate and starch do not correlate with any other properties, and hence are of little value in classifying this group. He confirms the basic division made by Smith and claims that dextrose, lactose, saccharose and raffinose constitute a true "metabolic gradient," and that fermentation of any one sugar implies fermentation of those preceding it in the series.

The work presented in this paper consists of a study of 80 organisms generally included in the colon group. Among the cultures studied were *B. coli communis*, *coli communior*, *acidi-lactici*, *coscoroba*, *aerogenes*, *capsulatus*, *pneumoniae*, *viscosus*, *cloacae*, *proteus* and *enteritidis*. These were all laboratory strains, at least 2 years old,

* Received for publication March 12, 1914.

1. *Centralbl. f. Bakteriol.*, 1895, 18, p. 494.

2. *Jour. Hyg., Cambridge*, 1905, 5, p. 333.

3. *Ibid.*, 1909, 9, p. 86.

4. *Jour. Infect. Dis.*, 1911, 8, p. 241.

5. *Systematic Relationship of the Coccaceae*, 1908.

6. *Science*, 1912, 35, p. 225.

isolated originally from a variety of sources: polluted water, feces, urine, the animal body, etc. The following tests were applied: morphology, Gram reaction; fermentation of dextrose, lactose, saccharose, raffinose, glycerin, salicin, mannite, dulcitol and inulin; action on milk and gelatin; reduction of nitrates; indol production, and the Voges and Proskauer reaction.

All the organisms studied were small uniformly staining rods (average 1.5μ . by 0.7μ .), reacting negatively to Gram, capable of fermenting one or more of the sugars (a few exceptions) with the production of either acid and gas, or acid alone. Of the 80 strains, 52 produced gas in both dextrose and lactose, 20 produced gas in dextrose and not in lactose, and 8 produced gas in neither. Of these 8, 5 produced acid but no gas in both of the sugars and will be included among the lactose fermenters, while 1 fermented dextrose and not lactose, and 2 failed to ferment at all. One of the last 2 came to us as *Bacillus mirabilis* and neither fermented nor liquefied and probably ought not to be classed with the proteus group at all; the other was sent to us as *Bacillus pneumoniae* and had either lost its fermentative properties or had been overgrown by another organism; no capsule could be observed. The dextrose fermenter is a cholera bacillus and was included with 2 others for comparison; the other 2 fermented with gas production, while this one did not ferment. It is also culturally different from the other 2, and really belongs to the septicemiae group. These 3 organisms will be excluded in the discussion of the results.

The fermentation tests were all conducted in standard meat infusion, sugar-free broth, to which 1 per cent. of the desired sugar was added. The cultures were incubated from four to five days at 37°C ., duplicate 5 c.c. samples were titrated with phenolphthalein as an indicator, and the average of the two results recorded. Controls were titrated but the initial acidity was not subtracted, as it was found that between certain limits the final acidity was a very constant quantity independent of the degree of initial acidity. This was shown to be the case by the following experiment: A few organisms selected at random from among those studied were inoculated into sugar broths of different acidities, varying from 0.5 to 3 per cent. normal acid, and titrated at various intervals. Five organisms and four of the test substances were employed. In all cases the acidity rose to a certain point which was quite constant for each organism in each sugar. The time required to reach the maximum acidity varied to some extent, of course, for the different organisms, but in most cases it was obtained in three, and rarely later than in four days. In the broth with the higher initial acidity there was a slight preliminary retardation, but the final result was about the same. The facts are summarized in Table 1.

A comparison between the acid and gas production in meat-infusion dextrose broth and a synthetic dextrose broth showed a decided varia-

tion in the latter. Many of the actively fermenting organisms in dextrose broth failed to ferment or fermented only feebly in the synthetic broth. This corresponds with Miss Broadhurst,⁷ who reported recently of the marked difference of acid production by streptococci in meat extract and meat infusion broth, respectively. Both these findings point out the importance of studying these organisms in a more or less uniform but always favorable environment. Under other conditions the results are not sufficiently constant to indicate the real relationship of the organisms studied.

TABLE 1
EFFECT OF INITIAL ACIDITY ON THE TOTAL FINAL ACIDITY

Sugar	Initial Acidity	Organisms Used				
		37	44	53	60	72
		Maximum Acidities				
Dextrose	0.6	3.3	5.1	3.8	3.2	3.8
	1.7	3.4	4.7	3.2	3.8	3.7
	1.9	3.8	4.8	4.0	4.0	3.7
	2.6	4.6	4.9	3.9	4.2	4.2
Lactose	0.6	3.5	4.5	5.3	5.2	5.2
	1.6	3.8	4.5	5.4	5.7	5.6
	2.0	3.7	4.5	5.0	5.5	5.1
	2.5	4.1	4.4	5.1	5.0	5.1
Saccharose	0.2	4.6	3.1	4.0	4.6	4.9
	1.5	4.7	3.6	4.7	5.2	5.1
	2.0	4.7	3.4	4.6	4.6	5.0
	2.4	5.0	3.4	4.6	4.7	4.8
Glycerin	0.2	3.1	2.1	3.6	4.0	2.5
	1.5	3.4	2.2	3.0	3.6	3.1
	1.8	3.6	2.0	3.3	3.2	3.6
	2.4	3.5	2.3	3.4	3.4	3.1

Indol was tested for in peptone broth after four days' incubation at 37 C. The Ehrlich test was found, after a careful comparative study,⁸ to be more reliable than the Salkowsky, and was used throughout. Nitrites were tested for in a nitrate-peptone broth in the usual way. Gelatin liquefaction was determined in small tubes 1 cm. in diameter, the depth of liquefaction after thirty days at 20 C. being recorded.

The fermentation reactions of the group are summarized in Figures 1 and 2. The most striking points brought out by these charts are the importance of quantitative titration of the acid produced and the unreliability of gas production, as ordinarily tested for as a distinguishing character. The work of Keyes and Gillespie⁹ shows a marked con-

7. *Jour. Infect. Dis.*, 1913, 13, p. 404.

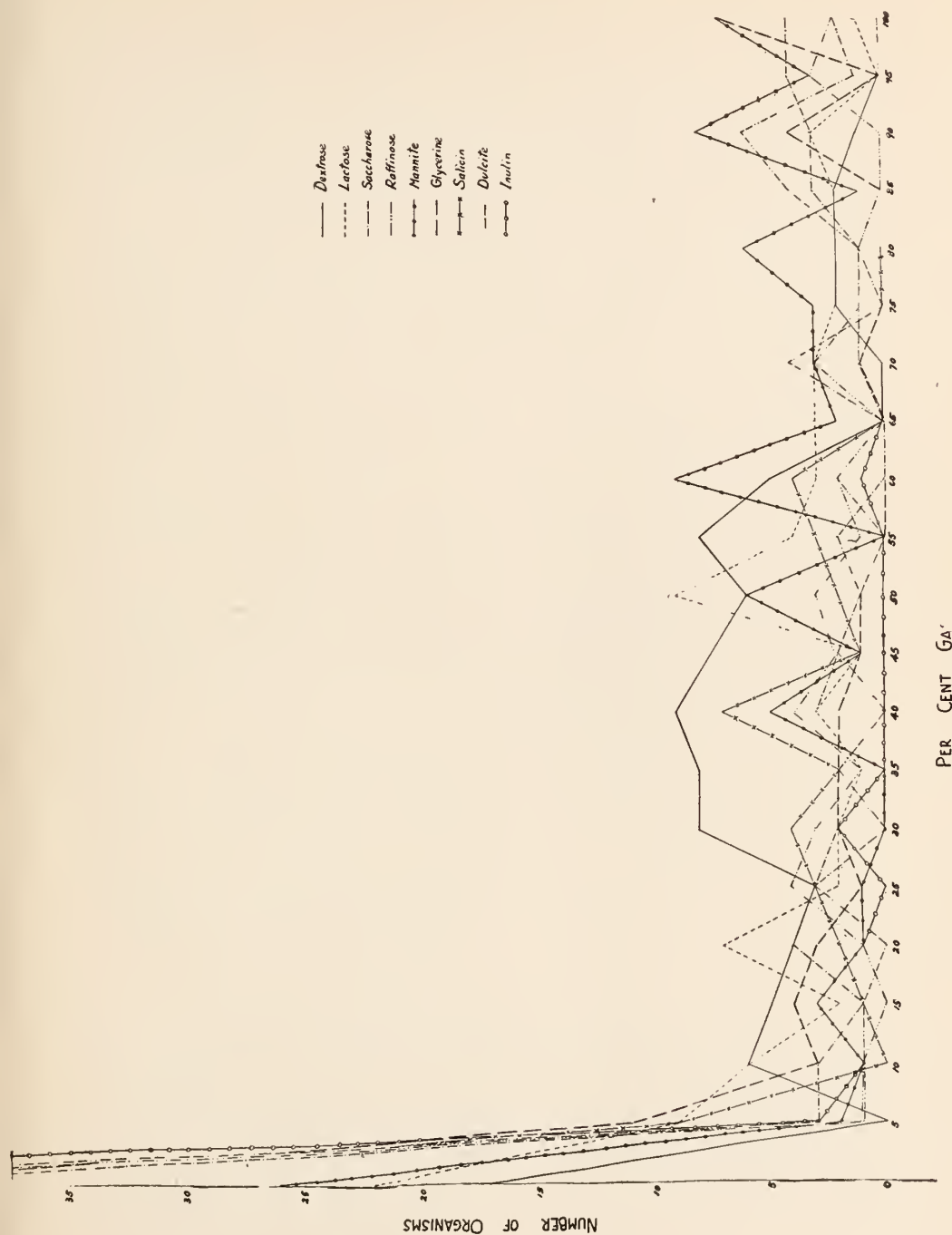
8. *Ibid.*, 1914, 14, p. 81.

9. *Jour. Biol. Chem.*, 1912, 13, p. 305.

stancy in the gas ratio when the test is carried out under carefully controlled conditions in a sealed tube. In the open tube, however, where solution and diffusion of the gases are constantly taking place, and where the physical factors of pressure and temperature exert such a decided influence on the phase equilibrium, there is no such constancy observed. It is doubtful, however, whether it is necessary to



FIG. 1.—Acid production in various sugars by members of the colon-typhoid group. Note the sharp break between fermenters and non-fermenters at 1.5 per cent normal acid.



PER CENT GA

Fig. 2.—Gas production in various sugars by bacteria of the colon-typhoid group. Note the absence of a sharp point of division.

employ the elaborate method of Keyes and Gillespie for testing gas ratios and gas quantities, when acid production gives such clear-cut differentiation when the acid is titrated quantitatively. Many organisms are capable of producing just enough acid to turn blue litmus red, but should not be counted as acid producers. As shown by Figure 1, the dividing line between acid and non-acid producers is very definite and generally falls at 1.5 per cent. normal acid. The neutral point for litmus is usually between 1.0 and 1.2 per cent. normal acid, depending on the grade. Therefore, while the litmus test may often be misleading, this is not the case when quantitative titrations are made with phenolphthalein as an indicator. The quantity of gas produced, on the other hand, fluctuates very much and gives no such regular curve as does acid production (Fig. 2). Many organisms too, while typical in every other respect, have apparently either partly or wholly lost their power to produce gas, judging by the names under which they were sent to us. The members of the proteus group, on the other hand, produced from 10 to 20 per cent. gas in lactose broth, though at no time did they produce more than 1.0 per cent. normal acid.

The extent to which the various sugars used are fermented (considering both acid, and acid and gas as positive) is of interest. Table 2 shows the order of availability of these substances for the entire group and for the lactose-fermenters only.

TABLE 2
SHOWING THE NUMBER OF ORGANISMS ATTACKING DIFFERENT SUGARS

Sugar Fermented	Dextrose	Mannite	Lactose	Glycerin	Saccharose	Salicin	Raffinose	Dulcitol	Inulin
All organisms studied	77	71	57	51	49	45	41	36	11
Lactose-fermenters only	57	57	57	48	41	39	38	28	9

The frequency with which the various sugars are fermented agrees in the main with the results obtained by Howe. Dextrose is fermented by all the bacteria tested. Next in order is mannite, followed by lactose. The first real division, as already well established, occurs in the fermentation of lactose. The colon-aerogenes-cloacae group is lactose-positive and the proteus-enteritidis group is lactose-negative.

Mannite seems of comparatively slight value for differential purposes, (1) because it is attacked by so large a proportion of the dextrose-fermenting strains (71 out of 77), and, (2) because it is not correlated with other properties,

the six non-fermenting strains belonging to the proteus group, with the exception of one which belongs to the enteritidis.

Considering for the present only the lactose-fermenting group, we note that glycerin and saccharose are the next substances most frequently attacked. The forms which ferment glycerin and not saccharose are scattered among many sub-groups and do not apparently represent a series of related forms. Saccharose, as Smith¹⁰ and Howe¹¹ have pointed out, seems to correspond to the first broad sub-division of the lactose-fermenting colon bacilli. In my own series, saccharose divides the group into two parts of approximately 70 per cent. and 30 per cent., respectively. Howe finds that saccharose divides his 540 lactose-fermenting organisms into groups of approximately 60 per cent. and 40 per cent., respectively. The agreement between my comparatively few, old stock, strains and Howe's freshly isolated bacilli is close enough to indicate that this is a uniform phenomenon.

Raffinose correlates so closely with the fermentation of saccharose that its importance as a classificatory substance is still debatable. Of the 57 lactose-fermenting organisms studied, 65 per cent. are saccharose-positive, raffinose-positive; 27 per cent. saccharose-negative, raffinose-negative; 5 per cent. saccharose-positive, raffinose-negative, and only 3 per cent. saccharose-negative, raffinose-positive. Howe gets 53 per cent., 41 per cent., 5 per cent. and 1 per cent., respectively. It is doubtful whether the 5 per cent. of aberrant strains deserve definite specific recognition. Winslow (1907)¹² has also found that fermentation of raffinose generally follows very closely that of saccharose. It is very likely that the same processes of hydrolysis and subsequent fermentation takes place in both cases. The sugars are very much alike chemically, in that neither reduces, and both yield fructose and glucose on hydrolysis.

Of the remaining sugars, inulin is fermented by only a few of the organisms (9), and of these, 8 belong to the aërogenes group. It does not, however, correlate with indol production or the Voges and Pros-kauer reaction, and does not appear to be of classificatory importance.

The substances left to be considered are salicin and dulcite. Of the two, dulcite is at present widely used to further differentiate the saccharose groups. It remains to be seen from an analysis of the results obtained whether or not it really correlates with other characters in such a way as to give a biological subdivision. Howe¹³ in the abstract of his results claims that dulcite does not correlate with any other character.

That the saccharose-positive group consists of more than one species is evident when one compares the saccharose-fermenting organisms now generally known as *Bacillus communior* and *Bacillus aërogenes*, respectively. The question is: Is the dulcite division biologically sound, and if not, what test or tests would give a better subdivision? Of the other tests performed, coagulation of milk and nitrate reduction are characteristic of the entire group, and hence of little classificatory

10. *Centralbl. f. Bakteriöl.*, 1895, 18, p. 494.

11. *Science*, 1912, 35, p. 225.

12. *Ibid.*, 1907, 26, p. 797.

13. *Ibid.*, 1912, 35, p. 225.

value. The Voges and Proskauer reaction is supposed to be given by the *aërogenes-cloacae* group only, and indol production is generally considered typical for the *communis-communior* group. Salicin, though found by Hilliard¹⁴ to be an important test substance for the streptococci, has not been employed for the classification of this group. Table 3 gives the correlation of indol production, Voges and Proskauer reaction, gelatin liquefaction and fermentation of glycerin with the dulcitate-saccharose and the salicin-saccharose groups, respectively.

TABLE 3

CORRELATION OF DULCITE AND SALICIN FERMENTATION RESPECTIVELY WITH OTHER CHARACTERS

		Organism	Per Cent Indol Positive	Per Cent V and P Positive	Per Cent Glycerin Fermenters	Per Cent Gelatin Liquefiers	Per Cent Salicin Fermenters
Saccharose-positive	Dulcitate-positive ...	20	50	20	85	5	50
Saccharose-positive	Dulcitate-negative ...	20	25	70	70	15	90
Saccharose-negative	Dulcitate-positive ...	9	100	0	100	0	89
Saccharose-negative	Dulcitate-negative ...	8	63	0	88	0	38
							Per Cent Dulcitate Fermenters
Saccharose-positive	Salicin-positive	28	21	68	68	14	35
Saccharose-positive	Salicin-negative ...	12	75	0	100	0	83
Saccharose-negative	Salicin-positive ...	11	100	0	100	0	73
Saccharose-negative	Salicin-negative ...	6	50	0	83	0	17

Table 3 shows that the saccharose-positive salicin-positive group corresponds closely with the saccharose-positive dulcitate-negative group. The former gives, however, a more definite division, in that it includes all the bacteria which liquefy gelatin and gives the Voges and Proskauer reaction. Again, unlike the dulcitate group, it is the only group which gives a large number of glycerin non-fermenters, a negative property which, as will be shown later, correlates with gelatin liquefaction and is characteristic of the *cloacae* group. Of the other groups, the saccharose-positive salicin-negative corresponds with the saccharose-positive dulcitate-positive; 83 per cent. of the former fermenting dulcitate; the saccharose-negative salicin-positive group agrees closely with the saccharose-negative dulcitate-positive, and the saccharose-nega-

tive salicin-negative group corresponds to the saccharose-negative dulcitate-negative.

Neither the saccharose-dulcitate nor the saccharose-salicin groups are sharply defined. It is, however, apparent from Table 3 that for the organisms studied salicin gives a more thoroughly biological classification, in that it correlates more closely with the other properties, and is also the more available of the two substances, as shown in Table 2. A sharp division of the groups on any basis could hardly be expected when one considers the large number of intermediary forms present among all groups of bacteria, and especially prevalent in this group.

A detailed comparison of the reactions of these 4 groups is given in Tables 4 to 7, inclusive; those headed "a" represent the dulcitate groups, while those headed "b" the salicin groups. The main features brought out by these tables have been summarized in Table 3. A few additional points are, however, worth mentioning. The salicin grouping, while it may include in the *aërogenes* group organisms which, though probably on the border line, belong rather to the *communior* division, it does on the other hand include all of the *capsulatus* and *pneumoniae* forms, which undoubtedly fall in the same group, if not in the same species, as does *Bacillus aërogenes*. This is not the case with the dulcitate classification, as Nos. 15, 37, 62 and 64 are obviously, both culturally and morphologically, members of the *aërogenes* group, and yet, according to their dulcitate fermentation, are classed with *Bacillus communior*. *Bacillus aërogenes* is generally decidedly different morphologically, from the other 3 species. The rods are, as a rule, shorter and thicker than the others; the growth on the agar streak is heavy, translucent and often viscid, instead of the flat almost transparent growth of *Bacillus communior*, etc., and in broth a thick pellicle is generally formed. These characters, though in themselves variable and of little differential value, are undoubtedly closely linked with the peculiar property of capsule formation manifested by this group, and while their absence does not necessarily exclude the organism from this species, as they are often lost on prolonged cultivation, their presence should certainly class them with *Bacillus aërogenes*. These points must not, of course, be pushed too far. They are merely indications of probable relationships which should be corroborated by more extensive studies with a larger number of organisms. It is also interesting to note that in the saccharose-salicin grouping practically all the forms derived from milk fall into either the *aërogenes* or the *lactici* groups

TABLE 4 (a)
SHOWING THE REACTIONS OF THE SACCHAROSE-POSITIVE DULCITE-NEGATIVE GROUP
(B. AEROGENES-MACCONKEY).

No.	Fermentation of Salicin	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
3	++	—	—	—	+	B. gasoformans	Water
7	++	—	—	—	+	B. viscosus	Milk
10	++	++	—	—	++	B. of ropy milk	Milk
36	++	++	++	—	—	B. aerogenes B2	Feces
39	—	++	++	—	—	B. aerogenes A1	Urinary fistula
40	++	—	++	+	+	B. aerogenes A2	Polluted well-water
48	++	++	++	—	—	Enterococcus	
50	++	—	++	+	+	B. cloacæ	Lockport canal
51	++	—	—	+	++	B. cloacæ	Intestine of sparrow
53	++	—	—	—	++	B. capsulatus	Kral
54	++	++	+	—	—	B. pneumoniae	
58	++	++	—	—	++	B. aerogenes	Lung
59	+	+	—	—	++	B. cloacæ	B. of A. In. 1897
60	+	+	—	—	+	Pfeiffer's b.	Original capsule bacil- lus of Pfeiffer
61	++	+	—	—	++	B. pneumoniae	
65	++	+	—	—	++	B. pneumoniae	Novy
66	++	+	+	—	—	B. aerogenes	Milk
72	++	++	—	—	++	B. capsulatus	
87	++	++	—	—	++	Paracolon b.	Milk
91	++	+	—	—	+	Paracolon b.	Milk

TABLE 4 (b)
SHOWING THE REACTIONS OF THE SACCHAROSE-POSITIVE SALICIN-POSITIVE GROUP
(B. AEROGENES-KLIGLER).

No.	Fermentation of Dulcitol	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
1	+	—	—	—	—	B. communior (lutea)	Feces
3	++	—	—	—	++	B. gasoformans	Water
7	++	++	—	—	—	B. viscosus	Milk
10	++	++	—	—	++	B. of ropy milk	Milk
15	++	++	+	—	—	B. of ropy milk	Ropy cream
33	++	++	—	—	++	B. coli communis	Dysentery stool
37	++	++	—	—	++	B. communior A1	Cystitis
40	—	+	+	+	+	B. aerogenes A2	Polluted well-water
48	—	—	—	—	—	Enterococcus	
50	—	—	—	+	+	B. cloacæ	Lockport canal
51	—	—	—	—	++	B. cloacæ	Intestine of sparrow
53	—	++	+	—	++	B. capsulatus	Kral
54	—	++	—	—	—	B. pneumoniae	
58	—	++	—	—	—	B. aerogenes	Lung
59	—	—	—	—	+	B. cloacæ	B. A. I. 1897
60	—	+	—	—	+	Pfeiffer's b.	Original capsule bacil- lus of Pfeiffer
61	++	+	—	—	+	B. pneumoniae	
62	++	++	—	—	++	B. aerogenes	Autopsy
64	++	++	+	—	++	B. aerogenes	Kral
65	++	++	—	—	++	B. pneumoniae	Novy
66	++	++	+	—	++	B. aerogenes	Milk
70	++	++	—	—	++	B. cloacæ	Milk
72	+	++	—	—	++	B. capsulatus	J. H. U.
87	++	++	—	—	++	Paracolon b.	Milk
90	++	++	—	—	—	Paracolon b.	Milk
91	++	++	—	—	+	Paracolon b.	Milk
88	++	—	—	+	—	Paracolon b.	Milk
89	++	—	—	—	—	Paracolon b.	Milk

TABLE 5 (a)
SHOWING THE REACTIONS OF THE SACCHAROSE-POSITIVE DULCITE-POSITIVE GROUP
(B. COMMUNIOR-MACCONKEY).

No.	Fermentation of Salicin	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
1	+	—	—	—	—	B. communior (lutea)	Feces
15	++	++	++	—	+	B. of ropy milk	Ropy cream
20	—	—	—	—	—	B. communior (rubra)	Water
27	—	++	++	—	—	B. communior A ₂	Feces
28	—	++	++	—	—	B. coli communis	Stool
30	—	++	++	—	—	B. coli communis	
33	++	++	++	—	—	B. coli communis	Dysentery stool
37	++	++	+	—	+	B. communior A ₁	Cystitis
41	—	++	++	—	—	B. communior C	Feces
43	—	++	++	—	—	B. communior B	Cystitis
44	—	++	—	—	—	B. coli communis	Cystitis
45	—	++	—	—	—	B. coli communis	Water
46	—	++	++	—	—	B. coli communis	Bird feces
47	—	++	++	—	—	B. coli communis	Milk
62	++	++	—	—	+	B. aerogenes	Autopsy
64	++	++	+	—	+	B. aerogenes	Kral
70	++	—	—	+	—	B. cloace	Milk
88	++	+	—	—	—	Paracolon b.	Milk
89	++	—	—	—	—	Paracolon b.	Milk
90	+	+	—	—	—	Paracolon b.	Milk

TABLE 5 (b)
SACCHAROSE-POSITIVE SALICIN-NEGATIVE (B. COMMUNIOR-KLIGLER)

No.	Fermentation of Dulcitol	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
20	+	++	+	—	—	B. communior	Water
28	++	++	—	—	—	B. communior A ₂	Feces
30	+	++	++	—	—	B. coli communis	Stool
36	—	++	++	—	—	B. coli communis	
39	—	++	++	—	—	B. aerogenes B ₂	Feces
41	++	++	++	—	—	B. aerogenes A ₁	Urinary fistula
43	++	++	+	—	—	B. communior C	Feces
44	++	++	—	—	—	B. communior B	Cystitis
45	++	++	+	—	—	B. coli communis	Cystitis
46	++	++	—	—	—	B. coli communis	Water
47	+	+	+	—	—	B. coli communis	Bird feces
						B. coli communis	Milk

(Tables 4 b and 7 b), while the communior and communis groups (Tables 5 b and 6 b) consist of those forms obtained from the animal body. This is not the case with the saccharose-dulcitate division. Another point worth noting is that the pathogenic forms, *Bacillus columbarum* and *Bacillus cholerae*, fall in the species of *Bacillus communis*.

TABLE 6 (a)
SHOWING THE REACTIONS OF THE SACCHAROSE-NEGATIVE DULCITE-POSITIVE GROUP
(*B. COLI COMMUNIS*-MACCONKEY)

No.	Fermentation of Salicin	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
23	+	++	++	—	—	<i>B. columbarum</i>	Kral
29	—	++	++	—	—	<i>B. coli communis</i>	Novy
31	++	++	++	—	—	<i>B. coli communis</i>	P. D. & Co.
32	++	++	++	—	—	<i>B. coli communis</i>	
35	++	++	++	—	—	<i>B. coli communis</i>	
38	++	++	++	—	—	<i>B. coli communis</i>	Feces
63	++	++	++	—	—	<i>B. coli communis</i> B.	Cystitis
79	++	++	++	—	—	<i>B. coli communis</i>	Autopsy
86	+	+	+	—	—	<i>B. cholerae</i>	Kral
						<i>B. cholerae</i>	Rahn

TABLE 6 (b)
SHOWING THE REACTIONS OF THE SACCHAROSE-NEGATIVE SALICIN-POSITIVE GROUP
(*B. COLI COMMUNIS*-KLIGLER)

No.	Fermentation of Dulcité	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
23	+	++	++	—	—	<i>B. columbarum</i>	Kral
31	++	++	++	—	—	<i>B. coli communis</i>	P. D. & Co.
32	—	++	++	—	—	<i>B. coli communis</i>	Rosenau
34	—	++	++	—	—	<i>B. coli communis</i>	
35	++	++	++	—	—	<i>B. coli communis</i>	Feces
38	++	++	++	—	—	<i>B. coli communis</i> B.	Cystitis
57	—	++	++	—	—	<i>B. aërogenes</i>	Intestine of Dog
63	+	++	++	—	—	<i>B. coli communis</i>	Autopsy
73	—	++	++	—	—	<i>B. acidilactici</i>	Kral
79	++	++	++	—	—	<i>B. cholerae</i>	Kral
86	+	+	+	—	—	<i>B. cholerae</i>	Rahn

The saccharose-positive salicin-positive group (corresponding to *Bacillus aërogenes*) may again be further subdivided into 2 subgroups, one fermenting glycerin and not liquefying gelatin, the other liquefying gelatin and failing to ferment glycerin. Of these two properties, that

of glycerin fermentation is the more reliable. Of the 28 organisms falling in this group, 9 failed to ferment glycerin. Four of these gave about 1 c.c. of liquefaction in 30 days. Two others were sent to us as liquefying organisms, No. 1 as *Bacillus communior-rubra*, liquefying in 26 days, and No. 59 as *Bacillus cloacae*, but have apparently lost their liquefying power. This has been frequently observed among cloacae organisms and is another reason why liquefaction is unreliable as a test.

TABLE 7 (a)
SHOWING THE REACTIONS OF THE SACCHAROSE-NEGATIVE DULCITE-NEGATIVE GROUP
(B. ACIDI-LACTICI-MACCONKEY)

No.	Fermentation of Salicin	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
34	+	+	+	—	—	B. coli communis	Feces Water Intestine of dog Milk Milk Milk Kral
55	—	++	++	—	—	B. acidi-lactici B.	
56	—	++	++	—	—	B. acidi-lactici A.	
57	+	++	++	—	—	B. aërogenes	
67	—	++	—	—	—	B. acidi-lactici	
68	—	—	—	—	—	B. acidi-lactici	
69	—	—	—	—	—	B. aërogenes	
73	+	+	+	—	—	B. acidi-lactici	

TABLE 7 (b)
SHOWING THE REACTIONS OF THE SACCHAROSE-NEGATIVE SALICIN-NEGATIVE GROUP
(B. ACIDI-LACTICI-KLIGLER)

No.	Fermentation of Dulcete	Fermentation of Glycerin	Indol Pro- duction	Gelatin Liquefaction	V. and P. Reaction	Original Name	Source
29	+	+	+	—	—	B. coli communis	Novy Feces Water Milk Milk Milk
55	—	++	++	—	—	B. acidi-lactici B.	
56	—	++	++	—	—	B. acidi-lactici A ²	
67	—	++	—	—	—	B. acidi-lactici	
68	—	—	—	—	—	B. acidi-lactici	
69	—	+	—	—	—	B. aërogenes	

Another strain (No. 3) was sent to us as *Bacillus gasoformans*, a liquefying organism, which failed to liquefy, but did not attack glycerin. Seven out of the 9 glycerin non-fermenting organisms are thus liquefying forms. The other 2 which neither liquefy nor ferment glycerin came to us as paracoli and are aberrant in several other respects. It may therefore be tentatively concluded that the saccharose-positive

salicin-positive glycerin-negative forms are a distinct group which corresponds to the cloacae group. This group is so closely related to *Bacillus aërogenes* that it has often been impossible to differentiate them. The glycerin test should therefore prove of value in this connection. Table 8 gives a summary of the reactions of this group.

The 20 organisms which ferment dextrose but fail to ferment lactose fall into 2 groups, consisting of 5 gelatin liquefying proteus forms, and 15 non-liquefiers.

All the 5 proteus forms ferment dextrose and saccharose and fail to ferment any of the other sugars. Two of the 5 ferment glycerin and fail to produce indol, while the other 3 produce indol but fail to

TABLE 8
CHARACTERISTICS OF THE BACILLUS CLOACÆ

Organism	Dextrose	Lactose	Saccharose	Raffinose	Dulcitate	Glycerin	Mannite	Salicin	Indol	Gelatin Liquefaction	V. and P. Reaction
40	++	+	+	++	+	—	+	++	—	++	+
50	++	++	++	++	++	—	++	++	—	++	++
51	++	++	++	++	++	—	++	++	—	++	++
59	+	++	++	++	++	—	++	++	—	++	++
70	++	++	++	++	++	—	++	++	—	++	++
1	++	++	++	++	++	—	++	++	—	++	++
3	++	++	++	++	++	—	++	++	—	++	++
89	++	++	++	++	++	—	++	++	—	++	++
91	+	+	+	+	+	—	+	+	—	—	+

* These came as liquefying organisms and have apparently lost that property under cultivation. None of the other characters has changed.

ferment glycerin. Four of these organisms (Nos. 11, 13, 14 and 17, see Table 9) came to us as *Bacillus vulgaris*, while No. 9 was sent to us as *Bacillus mirabilis*. From a study of 5 organisms it is, of course, impossible to determine whether the distinction is sufficiently important to separate them into 2 species. They may for the present be classed under *Bacillus vulgaris*. The distinctive characters of the species are summarized in Table 9.

The remaining dextrose-fermenters which failed to attack lactose are grouped in Table 10.

Table 10 points out that the organisms fall into two main divisions, one fermenting dulcitate and the other failing to do so. Strains 24, 42 and 71 are aberrant and cannot be grouped with the others. Strains 24 and 71 correspond with their type, *Bacillus coscoroba* and *Bacillus*

capsulatus, respectively, except for their inability to ferment lactose. Strain 42 is interesting. It was sent to us as *Bacillus communis*, but repeatedly failed to ferment any of the sugars besides dextrose. In the hope that a reversion would be caused, the bacillus was passed

TABLE 9
CHARACTERS OF THE *BACILLUS PROTEUS*

Name of Organism	No. of Organism	Dextrose	Lactose	Saccharose	Dulcite	Salicin	Indol	Glycerin	Gelatin Liquefaction	V. and P. Reaction
<i>B. mirabilis</i>	9	++*	—	++	—	+	—	+	++	—
<i>B. vulgaris</i>	11	+++	—	+++	—	—	—	—	—	—
<i>B. vulgaris</i>	13	+++	—	+++	—	—	—	—	—	—
<i>B. vulgaris</i>	14	+++	—	+++	—	—	—	—	—	—
<i>B. vulgaris</i>	17	++	—	++	—	—	—	+	++	—

* The reactions of this group as given above agree with those recorded by Jordan in 1908.¹⁵

TABLE 10
SHOWING THE REACTIONS OF THE GELATIN-NEGATIVE LACTOSE-NEGATIVE DEXTROSE-POSITIVE GROUP

No. of Organism	Saccharose	Raffinose	Dulcite	Mannite	Glycerin	Salicin	Milk	Indol Production	V. and P. Reaction	Original Name
24	+	+	+	+	+	+	+	+	—	<i>B. coscoroba</i>
42	+	+	+	+	+	+	+	+	—	<i>B. communis</i>
71	+	+	+	+	+	+	+	+	—	<i>B. capsulatus</i>
74	+	+	+	+	+	+	+	+	—	<i>Paracolon b.</i>
77	—	—	+	+	—	+	+	+	—	<i>B. enteritidis</i>
75	—	—	+	+	—	—	—	—	—	<i>B. enteritidis</i>
78	—	—	+	+	—	—	—	—	—	<i>Paracolon b.</i>
80	—	—	+	+	—	—	—	—	—	<i>B. enteritidis</i>
81	—	—	+	+	—	—	—	—	—	<i>B. enteritidis</i>
76	—	—	—	+	+	—	—	—	—	<i>B. enteritidis</i>
82	—	—	—	+	—	—	—	—	—	<i>B. cholerasuis</i>
83	+	+	—	+	—	+	—	—	—	<i>B. cholerasuis</i>
84	—	—	—	+	—	—	—	—	—	<i>B. cholerasuis</i>
85	—	—	—	+	—	—	—	—	—	<i>B. cholerasuis</i>
93	—	—	—	+	—	—	—	—	—	<i>B. suis</i>

successively through lactose broth for ten generations, with absolutely negative results. Of the other members of this group Strains 74 and 77 are identical, and though they fall in the dulcite-positive group they

differ from the others in fermenting salicin and producing indol, and should be classed by themselves as the paracolon bacilli. The remaining dulcitate-fermenters are alike in their reactions and conform to *Bacillus enteritidis*; three of the four strains came to us under that name. The dulcitate-negative forms were all (excepting Strain 76) obtained as *Bacillus cholera suis*, and with the exception of Strain 83, which seems to stand alone, all are characterized by the same fermentative properties.

No attempt was made to study the serum reactions of these organisms. Churchman and Michael¹⁶ have, however, worked out the agglutination reactions of a few of our strains which belong to this group, and a comparison of their results with mine sheds some light on the importance of fermentation reactions when the proper sugars are used and quantitative data recorded. These authors used those organisms which came to us as *Bacillus enteritidis*, corresponding with Nos. 77, 76, 75, 80 and 81 (Table 10) of mine. A summary of their results is given in Table 11.

TABLE 11
SUMMARY OF AGGLUTINATION REACTIONS (CHURCHMAN AND MICHAEL)

Organism	Paratyphoid	Sera E. 75	E. 81	E. 76	E. 77
B. paratyphosus	+++	0	0	0	0
E. 75	0	+++	++	0	0
E. 80	0	+++	++	0	0
E. 81	0	++	+++	0	0
E. 76	0	0	0	+++	0
E. 77	0	0	0	0	+++

Table 11 indicates that Strains 75, 80 and 81 are identical, and that Strains 76 and 77 differ from them as well as from one another. This result corresponds exactly with my results with the fermentation reactions, Strain 76 differing from 77, and both reacting in an entirely different manner from the other apparently true enteritidis organisms. A further study of such correlations between fermentation and sera reactions would be of great interest and should prove valuable in definitely establishing the species relationship of this very complex and ill-defined group.

SUMMARY AND CONCLUSION

Eighty organisms generally classed under the colon group were subjected to a series of fermentative and other tests with a view of determining their natural grouping as based on biometric principles.

Fifty-seven of these fell into the lactose-fermenting division; 20 did not ferment lactose, but fermented dextrose; 3 failed to ferment at all.

Acid production, as determined by titrating aliquot portions of the broth with phenolphthalein as an indicator, was found to be a more constant and a more reliable differential test than gas production, as ordinarily determined. The degree of initial acidity had no appreciable effect on the final acidity, which was quite constant, reaching its maximum on about the third day. The 57 lactose fermenters attacked mannite, glycerin, saccharose, salicin, raffinose, dulcite and inulin in the order named. Mannite, raffinose and inulin were considered to be of minor or doubtful classificatory importance. Saccharose divided the lactose group into two main subgroups.

On subdividing the saccharose groups, on the basis of dulcite and salicin fermentation respectively, it was found that the saccharose-salicin groups gave better correlation with indol production, Voges and Proskauer reaction and gelatin liquefaction than the saccharose-dulcite groups.

The saccharose-positive salicin-positive group (generally dulcite-negative) corresponds to *Bacillus aërogenes*.

The saccharose-positive salicin-negative group (generally dulcite-positive) corresponds to *Bacillus communior*.

The saccharose-negative salicin-positive group (generally dulcite-positive) corresponds to *Bacillus communis*.

The saccharose-negative salicin-negative group (generally dulcite-negative) corresponds to *Bacillus acidi-lactici*.

Glycerin was found to be of value in separating the cloacae forms from the aërogenes bacilli. Most of the saccharose-positive salicin-positive glycerin-negative group were gelatin liquefiers, indicating a reverse correlation between glycerin fermentation and gelatin liquefaction.

Of the dextrose fermenters, 5 of the organisms liquefied gelatin and fermented dextrose and saccharose, but failed to ferment any of the other sugars, with the exception of glycerin, which was fermented by 2 of the organisms. Of the other tests, all were negative with the exception of indol which was negative for the 2 glycerin-positive organisms and positive for the glycerin-negative bacteria. For the present all the 5 strains may be grouped under the *Bacillus vulgaris*.

The remaining dextrose fermenters which failed to ferment lactose and liquefy gelatin, were separated into three divisions: Those that

fermented dulcitol and salicin and produced indol, apparently corresponding with the paracoli bacillus; *Bacillus enteritidis* group which fermented dulcitol but neither attacked salicin nor produced indol, and *Bacillus cholerae suis* which fermented neither dulcitol nor salicin and failed to produce indol. A comparison of the fermentation reactions with some agglutination tests made by Churchman bears out this grouping. The main results are summarized in Figure 3.

We realize, of course, that this classification is based on a relatively small number of organisms and can at best be considered only tentative. The results are, however, sufficiently suggestive and interesting to deserve recording, and to merit further investigations along the

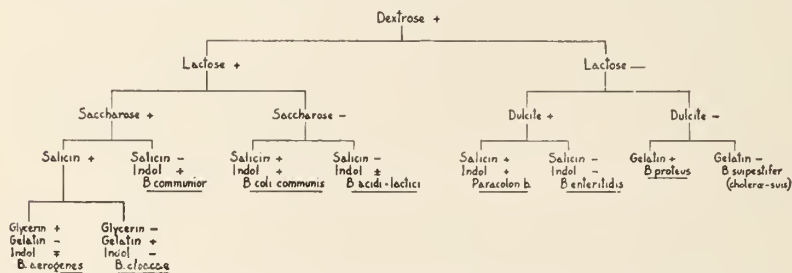


FIG. 3.—Diagrammatic representation of the salient characters of certain species of the colon-typhoid group.

lines indicated. From one viewpoint these results are valuable in that they are representative of a number of organisms of diverse origin, most of them, as far as we can tell, being isolated from distinct sources. In many studies a large number of strains are isolated from the same source, such as feces, water, etc. In these instances it is quite likely that about 50 per cent. of the strains from one source are but daughter cells of the same organism. A study of 100 forms does not, therefore, really represent the reactions of 100 distinct strains any more than 100 colonies, from a series of plates, sowed with a number of cultures of *Bacillus coli* would include as many different types. A study of the kind presented here is, therefore, of interest in that it gives the "mean" behavior of the groups, irrespective of the origin of the individual members belonging to it.

THE QUININ TREATMENT OF RABIES *

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The announcement by Moon¹ of the quinin treatment as a possible cure for rabies attracted much attention. His experiments, though few in number, were seemingly convincing. Shortly after Moon's announcement Harris² reported a case of successful cure by the administration of quinin in the human being. Since the report by Harris this treatment has been resorted to without success in at least three cases of hydrophobia in the human subject. As there were insufficient laboratory experiments on the quinin treatment for hydrophobia, and as this treatment was an almost universal failure when tried on human subjects, the following experiments are reported.

It was found that the dose of quinin tolerated by a full-grown rabbit was approximately 1 grain. Larger doses repeated daily were usually fatal, and even the 1-grain dose daily was fatal in several cases. For guinea-pigs the daily dose was 0.5 grain.

The quinin treatment was tested against not only the fixed virus, but also against street virus. The former was a six-day virus, while the latter killed rabbits in about ten days, and guinea-pigs in eight days. The virus was given intracranially; the bisulphate of quinin was used in all experiments, the injections being intraperitoneal.

In Experiment 1, five animals were injected intracranially with varying but small doses of fixed virus. The day following the inoculation three of the animals were injected intraperitoneally with 1 grain of quinin bisulphate. These injections were continued daily until death from rabies resulted. Animals 4 and 5 served as controls. Animal 3 died 15 minutes after the first injection of quinin. The symptoms preceding death were typical of quinin poisoning: rapid respiration, marked muscular tremor, unsteady gait, loss of equilibrium, convulsive seizure, and death usually within 45 minutes after injection. All the animals with the exception of Animal 3 died with the usual symptoms of rabies.

In Experiment 2, Animal 1 received no quinin until symptoms of rabies appeared, but the treatment had no appreciable effect. Animal 2 died of quinin poisoning 35 minutes after the second injection. Animal 3, treated with quinin, died from rabies.

Experiment 3 was a duplicate of Experiment 2. Animal 1, however, died from an acute bacillary infection and the quinin treatment had no preventive effect in Rabbits 2 and 3.

* Received for publication March 14, 1914.

1. *Jour. Infect. Dis.*, 1913, 13, p. 165.

2. *Jour. Am. Med. Assn.*, 1913, 61, p. 1511.

EXPERIMENT 1

Rabbit	Dose Fixed Virus Oct. 14	Quinin Treatment							
		Oct. 15	Oct. 16	Oct. 17	Oct. 18	Oct. 19	Oct. 20	Oct. 21	Oct. 22
1	1.05 M.I.D.*	1 grain	1 grain	1 grain	1 grain	1 grain	P. P. 1 grain	+
2	1.2 "	1 grain	1 grain	1 grain	1 grain	P. P. 1 grain	C. P. 1 grain	+
3	1.3 "	D
4	1.05 "	C. P.	+
5	1.2 "	P. P.	C. P.	C. P.	+

* Throughout the paper the following abbreviations are used: M.I.D. = the smallest infecting dose of rabie virus; P. P. = partial paralysis in rabies; C. P. = complete paralysis in rabies; D. = death from quinin poisoning; + = death from rabies.

EXPERIMENT 2

Guinea-Pig	Dose Fixed Virus Oct. 13	Quinin Treatment							
		Oct. 28	Oct. 29	Oct. 30	Oct. 31	Nov. 1	Nov. 2	Nov. 3	Nov. 4
1	1.0 M.I.D. D	P. P. 1 grain	C. P. 1 grain	+
2	1.05 "	1 grain	1 grain	C. P.
3	1.25 "	1 grain	1 grain	1 grain	1 grain	1 grain	1 grain	+

EXPERIMENT 3

Rabbit	Dose Fixed Virus Oct. 27	Quinin Treatment							
		Oct. 28	Oct. 29	Oct. 30	Oct. 31	Nov. 1	Nov. 2	Nov. 3	Nov. 4
1	1.0 M.I.D.	+
2	1.05 "	1 grain	1 grain	1 grain	1 grain	1 grain	P. P. 1 grain	C. P. 1 grain	+
3	1.25 "	1 grain	1 grain	1 grain	1 grain	1 grain	C. P. 1 grain	C. P. 1 grain	+

* Not rabies.

EXPERIMENT 4

Rabbit	Fixed Virus Subcuta- neously Oct. 27	Quinin Treatment	Nov. 18	Dec. 15
1	2500 M.I.D.	Daily injections (1 grain) until death	+	..
2	"	+

In Experiment 4 the fixed virus was given subcutaneously. Although the dose given is equal to 2,500 M. I. D. by the intracranial route, it is not always an infecting dose when given subcutaneously. Animal 1 was treated daily with 1 grain of quinin during the incubation period of 21 days; Animal 2 served as control. Both animals died from rabies.

Beginning October 28, two rabbits received eight daily injections of 1 grain of quinin without any untoward symptoms. On the ninth day each was injected with a small dose of rabic virus. The injections of $\frac{1}{2}$ grain of quinin were continued daily until death from rabies, on November 13, the treatment having had no appreciable effect. A similar experiment with guinea-pigs gave a like result. In this case the preliminary treatment extended over three days.

EXPERIMENT 5

Rabbit	Street Virus Nov. 12	Quinin Treatment	Nov. 23	Nov. 24	Nov. 27	Nov. 28
1	0.5 c.c.	Daily injections of 1 grain	P. P.	C. P.	+
2	0.5 c.c.	Daily injections of 1 grain
Guinea-Pig						
1	0.5 c.c.	Daily injections of 0.5 grain	P. P.	+
2	0.5 c.c.	Daily injections of 0.5 grain	+

The animals in Experiment 8 were injected intracranially with street virus and received during the incubation period daily injections of quinin. Rabbit 2 apparently did not become infected. Insusceptibility, though rare, does occur in animals injected even with fixed virus. The other three animals in this experiment died from rabies.

SUMMARY

The experiments were controlled both as to the infectivity of the virus and as to the absence, for the most part, of untoward symptoms from the quinin treatment.

The dose of virus in most of the tests was not much larger than the smallest infecting dose. This amount of the virus is approximately one-two-hundred-thousandth ($1/200,000$) part of the total virus in the brain and spinal cord of a rabbit at the beginning of the paralytic stage. In the case of hydrophobia in man we have no exact data as to the amount of virus, but it probably is many times greater than in the rabbit. Assuming that there is a quantitative reaction between the quinin and the virus, the protection — if any — afforded by large doses of quinin against a small infecting dose of virus should be easily demonstrated experimentally. Inasmuch as the quinin failed as a preventive measure against extremely small doses of virus in actual tests, can it not reasonably be assumed that this method of treatment is of no curative value in cases of hydrophobia manifesting symptoms

in which the amount of virus would be many thousand times greater? In the absence of protection by quinin against small doses of virus, even when a series of daily injections of quinin is given preliminary to the injection of rabic virus, we cannot expect favorable results from quinin after symptoms of hydrophobia have developed.

A METHOD FOR MAKING CARBOHYDRATE SERUM BROTH OF CONSTANT COMPOSITION FOR USE IN THE STUDY OF STREPTOCOCCI *

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For several years I have been studying the cultural and biological characters of the streptococcus group of the coccaceae, and have given particular attention to the development of the most favorable medium.

I learned very early in the work that in order to obtain good growth of the streptococci, fluid media were preferable to solid media. I also soon learned that the addition of serum to the media enhanced very markedly the development of the streptococci. It was often observed that exudate from appendiceal and other peritonitis cases showed in direct smears a mixture of Gram-negative bacilli and Gram-positive streptococci. Part of this material was added to plain broth and part to serum broth, and on examination of the growth in twenty-four hours or less the microscopical pictures from the two media were very different. That from the plain broth showed a predominance of Gram-negative bacilli with very few Gram-positive cocci, while the smear from serum broth showed what often appeared to be a pure culture of streptococci. From feces, one obtains practically the same results, that is, in the serum broth, the streptococci developing are numerous, while in plain broth they are almost absent.

That the addition of serum increases the growth of streptococci is, of course, well known. Elschmig¹ employed it in making cultures from the conjunctiva, and the high percentage (30) of streptococci which he obtained from apparently normal eyes is undoubtedly due to the use of the serum medium.

In studies on the pneumococcus the use of serum media was early recognized as necessary and was widely employed.

Hiss² used serum in his serum water media which were made as follows: Beef serum one part, distilled water two or three parts, heated in the Arnold sterilizer for 12 minutes at 100 C. One per cent. of a 5 per cent. aqueous solution of litmus was added until a deep transparent blue was obtained. One per cent. of the sugar to be used in the test was added to this medium. The serum water media were sterilized by the fractional method of 100 C.

* Received for publication March 15, 1914.

1. *Deutsch. med. Wchnschr.*, 1910, 26, p. 1229.

2. Hiss and Zinsser, *Text-Book of Bacteriology*.

Buerger,³ modifying these media by adding about 2 per cent. peptone, obtained better results in growing the pneumococcus than with Hiss' serum waters.

Ruediger⁴ further modified the serum water media. He employed beef serum which was diluted with an equal volume of water, passed through a large Berkefeld filter, heated to 65 C. for one-half hour on two successive days, and added in equal amounts to the previously tubed and sterilized litmus carbohydrate peptone and salt solution.

Buerger,⁵ in an article on the differentiation of streptococci, points out that the addition of ascitic fluid not only enhances growth but seems to favor the fermentation of certain of the carbohydrates that were not attacked in the simple media (sugar free broth), and lays emphasis on the use of the most favorable media for the growth of these organisms in doing the fermentation tests.

Heinemann⁶ used serum broth for "rejuvenating" the streptococcus lacticus before injecting it into rabbits. Marmorek used various strengths of different sera for producing rich and virulent cultures of streptococci, recommending equal parts of peptone broth and human serum as the best.

In an attempt to differentiate the large numbers of streptococci that were obtained in this laboratory carbohydrates were used as recommended by Gordon.⁷ The media used differed, however, in that the broth was made from Liebig's meat extract and serum added in the proportion of one part serum to four of the broth. The serum employed was usually hydrocele fluid but ascitic and ovarian fluids were also used. The two latter were very often found to be of little use in enriching our media. All three sera were moreover quite inconstant in their albuminous content and the results obtained were, at times, quite irregular. This led to the attempt to make a serum broth on which one could rely for always obtaining a good growth of streptococci. After we had tried several of the serum waters and found them, although a decided improvement on plain broth, not as satisfactory as the serum broth, the following serum medium was prepared which was later improved.

Beef blood was collected from the abattoir in sterile quart jars, such as are commonly used for storing preserves, called lightning jars. The blood was allowed to clot in the cool room of the abattoir for about 15 minutes. It was then brought to the laboratory, rimmed, and allowed to stand overnight in a cool place. The clear serum was obtained by centrifugalization, after which it was ready for use. One hundred c.c. of this serum was added to 300 c.c. of distilled water, and sterilized for 15 to 20 minutes in flowing steam on three successive days. The mixture became slightly milky or opalescent but was perfectly transparent.

3. *Jour. Exper. Med.*, 1905, 7, p. 524.

4. *Jour. Infect. Dis.*, 1906, 3, p. 756.

7. *Ann. Rept. Loc. Gov. Bd., London*, 1903-4, p. 388.

5. *Jour. Exper. Med.*, 1907, 9, p. 428.

6. *Jour. Infect. Dis.*, 1907, 9, p. 87.

The carbohydrate broth was made as follows:

Peptone (Witte)	40 gm.
Meat extract (Liebig's)	12 "
Sodium chlorid	20 "
Distilled water	1,000 c.c.

This broth was four times the usual strength. It was made neutral to phenolphthalein (hot titration) and 4 gm. of the carbohydrate and 4 c.c. of Andrade's indicator were added to 100 c.c. This broth was sterilized in flowing stream for 15 to 20 minutes on three successive days.

The sterile serum water was then mixed with this quadruple strength carbohydrate broth, and the medium, consisting of 1 part serum to 4 parts 1 per cent. carbohydrate broth, was tubed into sterile tubes by means of a sterile tubing-funnel, or by use of a sterile syphon. The use of the syphon was as follows: The 500 c.c. of carbohydrate serum broth was mixed in a large flask. A rubber cork was fitted with a syphon and a glass tube bent at right angles, the outer end of which was plugged with cotton wool. This apparatus was sterilized in another flask, transferred to the flask containing the medium, the syphon started by blowing through the bent tube, and the medium tubed into sterile tubes.

This medium gives a much better growth of streptococci than the serum waters or ordinary broth. It is coagulated on the production of acid in the fermentation of the carbohydrates as is the case in all heated serum media (Longcope).

The heating of the serum undoubtedly alters many of its albuminous constituents, possibly they are changed into the so-called colloidal state as Longcope⁸ suggests. Whatever the change, it is not as favorable a medium for streptococcus growth as the unheated serum.

In order, therefore, to obtain a medium containing unheated serum it was decided to sterilize the serum by filtration. It was found, however, that the filter became clogged after a relatively small amount of the serum had passed through. To overcome this, the serum was diluted one-half with distilled water and it now passed without any difficulty through an ordinary Berkefeld filter. To assure sterility, it is important that the filtration takes place slowly as otherwise organisms pass through the filter candle.

A very useful addition to the ordinary filtering apparatus is the insertion of a large glass tube into the rubber cork of the filtering flask to direct the filtered serum past its side opening. Also a large inverted test tube covering the filter candle makes possible the filtration of the last of the serum without the bubbles that are commonly formed.

The carbohydrate broth with which the serum was subsequently mixed was made up as follows:

Double strength broth + 1.2 ac.....	200 c.c.
Distilled water	100 c.c.
Carbohydrate	4 gm.
Andrade's Indicator	4 c.c.

Sterilized in flowing steam 15 to 20 minutes on three successive days, cooled and then 200 c.c. of the diluted and filtered serum added.

The finished medium gave a carbohydrate serum broth. It was tubed as above described and was incubated for several days before use to ensure sterility.

Different strengths of serum are easily obtained by varying the above formulae. The dilute serum obtained as described above is very useful to add to carbohydrate agar for anaerobic cultures.

The advantages of this method for making serum broth are: (1) A uniform mixture is obtained in all tubes; (2) there is less liability for contamination than by the use of sterile pipets and the addition of the serum to each tube; (3) the serum used has never been heated and is, therefore, unaltered; (4) the use of beef serum assures a serum of reasonably constant composition and it is, therefore, useful for comparative tests. Moreover, as Longcope⁹ has pointed out, beef serum does not show the production of acid with the growth of pneumococcus and in my experience acid is never produced in the control sugar-free serum broth with a great variety of streptococci; (5) this serum medium is not coagulated by the production of acid, only a slight opalescence appearing when much acid is produced.

I have carried out a number of experiments with the streptococcus by growing it in ordinary carbohydrate broth and in the carbohydrate serum broth, and our results have been striking. I have found that many strains of streptococci fail to grow at all while others grow very poorly in ordinary broth. It is true, on the other hand, that a great number grow well in both media, but always more luxuriantly in the serum broth. It is, I believe, fundamental in the study of the fermentation reactions of the streptococci, to have a medium in which the organisms grow well independently of the carbohydrate added. Although many of the streptococci will ferment a certain carbohydrate in broth, serum water, or serum broth, others grow so poorly in the former that they fail to attack the carbohydrate, and the result would appear negative if no further study were undertaken.

In a recent article Broadhurst¹⁰ has shown that she gets a higher acidity in meat than in meat extract broth. It is well known that meat infusion broth makes a better medium for sensitive organisms than meat extract broth, and I would be inclined to believe that she was dealing with a more vigorous growth in the former case.

Floyd and Wolbach¹¹ on the differentiation of streptococci say, "We are not certain that the clotting of milk is solely dependent upon carbohydrate fermentation. This is tentatively offered in the light of our experience in that milk may be acidified without acid production in dextrose and lactose serum waters." It is interesting to note in this connection that out of 63 strains in their Group 2, 29

9. *Ibid.*

10. *Jour. Infect. Dis.*, 1913, 13, p. 404.

11. *Jour. Med. Research*, 1914, 29, p. 493.

show acid in milk and 34 clotting of milk without producing any acid in lactose. In Group 4, they found that out of 42, 8 produced acid and 3 clot in milk, without acid in lactose serum water. In Group 5, out of 43, only 1 produced clot in the milk without acid in the lactose medium. In Group 6, the results in milk and in lactose serum water are the same with the exception that two strains failed to affect either the milk or the lactose serum water.

Group 2 "corresponds closely to streptococcus pyogenes." Group 4 is composed of non-hemolytic strains and is "intermediate between streptococcus pyogenes and the streptococcus anginosus." Group 5 "corresponds closely to streptococcus anginosus."

The more strictly parasitic streptococci are grown with greater difficulty in artificial media and I would suggest for these confusing results the explanation that the growth in the lactose serum water is not vigorous enough to allow the organisms to exert their fermentative powers, while in the milk the conditions for growth are more favorable and the organisms ferment the contained lactose and dextrose. The explanation of the results of Group 1, where no fermentative action was demonstrated or only acid was developed in dextrose, is more difficult as milk is not affected. "These cultures come mostly from cases where the streptococcus played a pathogenic role." I have met in investigations of about 500 strains a fair number (25) of streptococci which failed to ferment lactose, but which hemolysed blood. They are similar to the 44 out of 62 strains observed by Floyd and Wolbach. I have also encountered a few strains (12) which do not hemolyse and which correspond to the streptococcus equinus of Andrewes and Horder.¹² However, I have always noted a slight acidity in litmus milk and acid was formed in the dextrose and saccharose serum broth. The five strains in Group 3 and one in Group 5, as reported by Floyd and Wolbach,¹³ which appear to ferment the lactose in the serum water media but fail to affect any change in milk, are interesting and would bear further investigation.

Winslow and Palmer,¹⁴ in a comparative study of intestinal streptococci, "utterly failed" to isolate streptococci from feces by growing in dextrose broth preliminary to plating on agar and resorted to plating on agar directly, which method "proved generally successful."

From my experience, I feel certain that the use of serum broth would have materially helped them in their isolations. These workers also had in their carbohydrate broth fermentation tests a considerable number of "clear tubes" in which no obvious growth of the streptococci had occurred. These tubes were plated out on agar with the result that out of 49 tubes plated "3 showed many colonies, 15 showed 1 to 6 colonies, and 31 showed none." "It may reasonably be assumed," they say, "that in such cases the streptococci introduced had simply failed to develop and gradually died out on account of the lack of suitable carbohydrate pabulum, on which these organisms appear to be highly dependent." It would seem that these streptococci which failed to develop and others in which feeble growths had occurred had not been given the most favorable environment in which they could demonstrate their fermentative powers.

Salomon¹⁵ and other German workers in studying the fermentative powers of the streptococci have used litmus ascites carbohydrate agar. Their results are very different from those obtained by others where fluid media were employed. Salomon found that 13 strains of the pneumococcus and 6 out of 10 of his strains

12. *Lancet*, 1906, 2, p. 708.

13. *Jour. Med. Research*, 1914, 29, p. 493.

14. *Systematic Relationships of the Coccaceae*.

15. *Centralbl. f. Bakteriologie*, Abt. I, Orig., 1908, 47, p. 1.

of streptococcus mucosus practically failed to show any fermentative powers by his method, although he used 18 different carbohydrates. These organisms are well known to have high powers of fermentation, and the results Salomon obtained are due, we believe, to the use of solid in preference to fluid media.

Winslow¹⁶ in his study of the coccaceae draws attention to the fact that in obtaining material by the method of plating on agar and incubating at 20 C. he failed to obtain many of the more strictly parasitic streptococci which grow only feebly on solid media and are most active at a temperature of 37 C. In this I agree perfectly, but would go further and say that I believe a study of streptococci without the use of serum broth loses much of its value, from failure to isolate many of the less easily grown strains, and furthermore, many are not given a sufficiently rich medium in which they may develop vigorously enough to exercise their fermentative action on the contained carbohydrate.

There is a well recognized difference in cells in respect to their ability to exercise their specific functions. These functions may be entirely absent where the cells are merely living or even slowly reproducing. A healthy state of the cell where both reproducing and functional activities are at their highest is to be found only where the environment is best suited to the needs of the cell.

This is well known when we consider the functional activity of the cells in the metazoa and is, I believe, equally true among the unicellular organisms now under discussion.

CONCLUSIONS

Serum broth is the most favorable medium for the isolation and growth of streptococci. It is so well suited to the growth of streptococci that in mixed cultures, even vigorous organisms such as the bacillus coli are overgrown in twenty-four hours.

Cultures containing different forms of streptococci should be planted in serum broth for at least twenty-four hours before plating on blood agar. If this is not done, many of the more pathogenic forms are liable to be overlooked.

Many strains of streptococci grow poorly in plain carbohydrate broth while others fail to show any growth at all.

In testing the fermentative powers of streptococci the carbohydrates should be added to serum broth.

The method here described offers a means for making a carbohydrate serum broth of reasonably constant composition, and which, in my hands, has always given a good growth of streptococci.

16. Systematic Relationships of the Coccaceae.

THE ACTION OF VACCINES AND OF CONCENTRATED ANTISTREPTOCOCCUS SERUM IN EXPERIMENTAL STREPTOCOCCAL ARTHRITIS *

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EXPERIMENTS WITH CONCENTRATED ANTISTREPTOCOCCUS SERUM

Antistreptococcus serum has been employed for therapeutic purposes for several years without altogether satisfactory results. Since there is frequently evidence of the presence in such sera of antibodies, it is possible that low potency may be an important factor in explaining the unsatisfactory results. For this reason it was thought advisable to make some experiments with concentrated serum.

In the preparation of the serum, part of which was done by Dr. L. C. Gatewood, fifty-two strains of streptococci, including viridans and hemolyticus, as well as strains from cases of epidemic sore throat, were injected into horses. The strains were isolated from patients having tonsillitis alone or complicated with acute or chronic arthritis, endocarditis, serositis, nephritis and pyorrhea. The virulence of many strains were tested on rabbits; thirty-four produced acute or chronic arthritis in these animals.

Four horses were used,¹ three being injected with a mixture of various types of streptococci, the fourth receiving only strains from epidemic sore throat. After each strain grew for twenty-four hours on a blood agar slant, it was washed off with sterile normal salt solution, the suspension mixed in a large test tube, a drop or two of chloroform added to this, and the whole heated for sixty minutes at 55 C. After heating tests were made to insure sterility. The suspension was then measured and the required quantity taken for injection. The first doses were equal to the growth of one blood agar slant; subsequently they were increased, depending on the reaction of the horse.

Horses 1, 2 and 3, were given a suspension from thirty-seven strains of streptococci, nineteen of which had produced arthritis in

* Received for publication April 9, 1914.

1. This work was made possible through the generous cooperation of the Memorial Institute for Infectious Diseases, which gave the facilities for the production of immune serum. I am also indebted to Dr. P. G. Heinemann for advice and assistance in the concentration of the serum.

rabbits. After several doses of dead organisms increasing from one to six slants, Horses 1 and 2 received doses of from one-tenth to one slant of living cocci. The larger doses of these caused local abscesses with such unsatisfactory results that this method of immunization was discarded. It had been previously found by Heinemann and Gatewood² that when doses of from ten to fifteen slants of killed streptococci were injected, the opsonic index of the serum soon reached from 1.8 to 2.5, and he therefore used this quantity of organisms for immunization. The injections were made at intervals of ten days.

Horse 4 was immunized with fifteen strains of the epidemic type of streptococcus. Most of these were isolated from the Chicago epidemic, but organisms from the Boston, Rummelsburg (Germany), and Baltimore epidemics were included in the number. The vaccine was prepared and given as described.

Practically the only reaction noticeable in the horses was a rise in temperature of from 1 to 5 F. after the injection. This was most marked after the earlier injections, especially the first two or three doses of ten slants. Occasionally a horse suffered from stiffness of the joints after an inoculation. Bleedings were made about once a month; 2 gallons were obtained each time. The blood serum was then concentrated and titrated according to the method described by Heinemann and Gatewood.³ They found that the antibodies of anti-streptococcus serum are attached to the globulins similar to the antitoxins of antidiphtheria serum. Precipitating and dialyzing the globulins by the methods of Gibson and Banzhaf in concentrating antidiphtheria serum, they increased the potency of the concentrate from 3 to 5.6 times the potency of the original serum, as measured by the opsonic index. The opsonic index before concentration ranged from 1.5 to 2.8, after concentration from 5.3 to 13.3. I obtained similar results.

In order to test the efficiency of the serum rabbits were used, since it has been demonstrated by several investigators⁴ that an acute or chronic arthritis is produced readily in these animals. After a few preliminary tests, a streptococcus was found which, in relatively small doses, almost never failed to produce acute arthritis three to five days after an intravenous injection. This streptococcus was isolated from

2. *Jour. Infect. Dis.*, 1912, 10, p. 416.

3. *Ibid.*

4. Walker, *Brit. Med. Jour.*, 1903, I, p. 237; Combs, Miller and Kettle, *Lancet*, 1912, 2, p. 1209; Poynton and Paine, *Lancet*, 1902, 2, 861; Davis, *Jour. Am. Med. Assn.*, 1912, 58, p. 1283; *Jour. Infect. Dis.*, 1912, 10, p. 243; Jackson, *Ibid.*, 1913, 3, p. 364; and others.

the tonsils of a man, aged 42 years, suffering from chronic arthritis of one year's duration. The patient had attacks of febrile exacerbation every four to six weeks, accompanied by swelling of the wrist, shoulder, ankle and knee joints. The heart and kidneys were normal. The tonsils were moderately enlarged but not acutely inflamed. A hemolytic streptococcus (256) was isolated in almost pure culture from the crypts of both tonsils. A dose of this streptococcus of one-twelfth to one-tenth of a twenty-four-hour growth on one blood agar slant, approximately 100,000,000 to 125,000,000 streptococci, on intravenous injection produced joint involvement in 95 per cent. of the rabbits in three to five days. The other 5 per cent. usually died of septicemia within about four days.

The antistreptococcus serum was injected intraperitoneally in amounts varying from 5 to 20 c.c. This route was selected since the absorption would be more rapid than by subcutaneous administration, and the dangers attending intravenous injection could be avoided. Half-grown male rabbits were found to be most suitable. The experiments in rabbits fall into four sets:

1. Rabbits treated with serum after developing acute joint infections, following the injection of streptococci.
2. Those treated with serum immediately following the injection of the streptococci and at intervals until the recovery or death of the animal.
3. Those treated with serum before the injection of the live organisms.
4. Those treated with serum before and again after the injection of streptococci.

I. ANIMALS TREATED WITH SERUM AFTER DEVELOPING ACUTE ARTHRITIS

Of the rabbits treated with serum after the appearance of arthritis but one survived. These rabbits received doses of serum ranging from 5 to 20 c.c. on various days. Brief protocols of some of the experiments follow:

Rabbit 3.—Injected intravenously with 1/10 tube of *Streptococcus* 256. Left wrist swollen and inflamed on the third day; 10 c.c. of serum intraperitoneally on third day. Death on fourth day.

Rabbit 16.—Injected with 1/10 tube of *Streptococcus* 256. Left ankle involved on sixth day. Given 20 c.c. serum on seventh day, 15 c.c. on eighth day. Died on ninth day.

Rabbit 17.—Injected with 1/10 tube Streptococcus 256. Left elbow swollen on third day. Injected with 20 c.c. serum on the third, fifth, seventh and eighth days. Died on the ninth day.

Rabbit 23.—Injected with 2 tubes Streptococcus Raven. Left wrist swollen on fourth day, followed by swelling of the right wrist on the fifth day. Given 10 c.c. serum on the fifth and sixth days, and 5 c.c. on the seventh day. Died on the seventh day.

Rabbit 30.—Injected with 1/10 tube Streptococcus 256. Left ankle joint involved on seventh day, followed by swelling of right hip. Serum administered in 6 c.c. doses on the fourth, eighth and tenth days. Rabbit died on fifteenth day. Decreased in weight from 4 lb. 8 oz. to 3 lb. 2 oz.

Rabbit 9.—Injected with 1/8 tube Streptococcus 256. Right wrist affected on third day. Serum administered as follows: 10 c.c. fourth day; 20 c.c. each on fifth, sixth, seventh, eighth, tenth, eleventh, fourteenth and nineteenth days; 15 c.c. on twenty-third day; 7 c.c. on twenty-seventh day; 6 c.c. on twenty-eighth day; 10 c.c. on twenty-ninth day; in all, 220 c.c. of serum. Died on the seventy-fifth day.

Rabbit 10.—Control: Injected with 1/8 tube of Streptococcus 256. Left wrist and knee involved on third day. Death on sixth day.

Rabbit 13.—Control: Injected with 1/10 tube of Streptococcus 256. Right wrist swollen on second day. Died on fifth day.

Rabbit 11.—Control: Injected with 1/8 tube of Streptococcus 256. Left shoulder involved on second day. Killed on sixty-eighth day. The joint was swollen but firm at that time.⁵

Rabbit 9 was the only rabbit in this group that recovered from the acute infection. This animal ran a chronic course which ended eventually with a malformed joint. However, the control, Rabbit 11, passed through a similar clinical course with the involvement of but one joint. In none of the nine rabbits treated with the antistreptococcus serum after the development of acute arthritis were any beneficial results apparent. The course of the disease in these was similar to that of the untreated animals. The antibodies, using the opsonic index as a guide, were not increased and death occurred at a shorter period in treated than in untreated animals.

II. ANIMALS TREATED IMMEDIATELY FOLLOWING THE INJECTION OF THE STREPTOCOCCI AND AT INTERVALS UNTIL THEIR RECOVERY OR DEATH

A brief description of two rabbits will show the action of the serum:

Rabbit 14.—Injected with 1/10 tube of Streptococcus 256, November 26. Given 10 c.c. of serum immediately, and 10 c.c. daily until death on the tenth day. On the third day the tarsal joints of the right forefoot were swollen; on

5. The anatomy of this joint with microphotograph can be found in the article on "Experimental Streptococcal Arthritis in Rabbits," by Jackson, *Jour. Infect. Dis.*, 1913, 12, p. 377.

the fourth day the right knee and left wrist. The joint lesions increased in severity. By the ninth day the rabbit had received 90 c.c. of serum.

Rabbit 18.—Injected with 1/10 tube of Streptococcus 256. Given 10 c.c. of serum immediately and 10 c.c. daily for the first six days. The left wrist became affected on the third day, the left knee and ankle on the fourth day, the right elbow on the fifth. Death occurred on the ninth day.

Rabbit 24.—Control: Injected with 1/10 tube of Streptococcus 256. The left ankle was involved on the second day, the right ankle on the fourth day, both wrists on the sixth day. Death occurred on the tenth day.

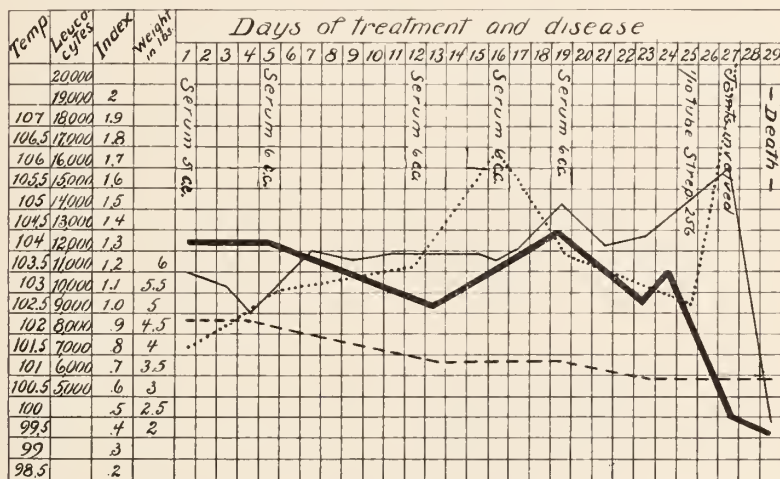


Chart 1.—Prophylactic treatment with serum. Rabbit 35.

Heavy solid line—Opsonic index

Dotted line—Leukocyte count

Fine solid line—Temperature

Fine broken line—Weight curve

Although the treated rabbits received 90 and 60 c.c. of serum, the control animal lived twenty-four hours longer. It might be supposed that these rabbits were overwhelmed by the serum, but the administration of similar amounts to control animals showed nothing but a slight decrease in weight. The rabbits weighed on an average about 2,000 gm. On this basis the amount of serum received daily (10 c.c.) would be equivalent to over 300 c.c. daily for a man weighing 75 kilos (150 pounds). In none of the four rabbits used was serum found in the peritoneal cavity at necropsy. The results in this group are similar to those of the first, and show that the administration of the serum did not increase the resistance of the animals.

III. ANIMALS TREATED WITH SERUM BEFORE THE INJECTION OF THE STREPTOCOCCI

To test the serum as a prophylactic agent against streptococcus infections, it was administered to four rabbits in doses varying from 5 to 20 c.c. at two- and four-day intervals, over a period of two weeks preceding the injection of *Streptococcus* 256. From the experiments it was clearly evident that no protection was afforded by serum, the joints becoming involved usually on the fourth day, with death of the animals by the ninth day. Protocols of two of the rabbits are here given:

Rabbit 34.—February 5, 10, 17, 21 and 24, injected with 5 or 6 c.c. of serum. On March 1, it was injected intravenously with 1/10 tube of *Streptococcus* 256. Right ankle was swollen and tender by the fourth day. Death occurred on the ninth day.

Rabbit 35.—February 5, 10, 17, 21 and 24, given 5 and 6 c.c. of serum. On March 1, was injected with *Streptococcus* 256. Right wrist and left ankle swollen on the fourth day. Death occurred on the fifth day.

Rabbit 37.—Control: Injected with 1/10 tube of *Streptococcus* 256, March 1. Both ankle joints swollen by the fourth day. Death occurred on the ninth day.

IV. ANIMALS TREATED WITH SERUM BEFORE AND AGAIN AFTER THE INJECTION OF THE LIVING STREPTOCOCCI

In this group antistreptococcus serum was administered before and after the injection of the living streptococcus. The four animals treated showed no improvement over the control animals.

Since *Streptococcus* 256 was one of the organisms in the polyvalent vaccine with which the horses were injected in the preparation of the serum, most of these rabbits received an autogenous as well as a polyvalent antistreptococcus serum.

Three streptococci other than 256 were used in producing arthritis to test the efficiency of the serum. The technic was the same as that described, except that the doses of the live organisms were larger, the twenty-four-hour growth on one to three blood agar slants being the usual amount given. Serum was administered in 5 to 20 c.c. doses daily, and at longer intervals. All the animals developed acute arthritis, and died on or before the ninth day.

A chart of a rabbit which had prophylactic injections of serum is given. This chart is selected because the experiments extended over a length of time sufficient to give one a fair idea of the action of the serum on animals, and because the reactions here are typical of those

of other groups. Taking up the opsonins first, we find that in Rabbit 35, the variations are almost identical with those of the control animal, Rabbit 36. Treatment with serum after injection of the living streptococcus showed no changes in opsonins different from those shown on the chart, there always being found a rapid decrease.

There was a gradual loss in weight during the entire period of treatment. This is important, as the control animals during the period preceding injection of the living streptococcus almost always gained in weight. After introduction of the live streptococci, there was in almost all cases a more or less rapid fall in the weight curve. The leukocyte curve was variable; on the whole, there was a gradual increase in the leukocytes during the prophylactic treatment, with a sudden rise in all cases after receiving the living organism. In some cases this went as high as 50,000 leukocytes per cubic millimeter. The temperature curve before the injection of *Streptococcus* 256 is erratic, and is not worth much as a guide to the extent of any serum reaction. There is always a marked rise in temperature after the injection of the live organism reaching nearly 106 F., in some instances with a rapid fall usually immediately before death.

The same concentrated serum, combined with treatment with autogenous vaccines, was employed by Dr. Frank Billings⁶ in a series of cases of chronic arthritides. The serum appeared to heighten the immunizing power of the autogenous vaccines, but anaphylactic reactions were so severe that it had to be discarded. In no case was the serum used as the only treatment. As the serum alone had apparently no immunizing or curative qualities, it was deemed advisable to ascertain the effects of vaccines alone.

EXPERIMENTS WITH STREPTOCOCCAL VACCINES

In the experiments I endeavored to immunize rabbits with repeated doses of homologous streptococcal vaccines, followed by an injection of living streptococci known to produce arthritis in control animals.

The technic was as follows:

The rabbits were injected at intervals of two to four days with five or six doses of vaccine prepared from the organism, which later was injected to produce the arthritis. The injections were made subcutaneously on both sides of the vertebral column. The first dose of vaccine was 500,000,000, and this was gradually increased so that the last one contained 1,000,000,000 to 2,000,000,000 streptococci. Three or four days after the last injection the rabbits were injected intravenously with 1/10 of the twenty-four-hour growth on a blood agar slant

6. *Jour. Am. Med. Assn.*, 1913, 61 p. 821.

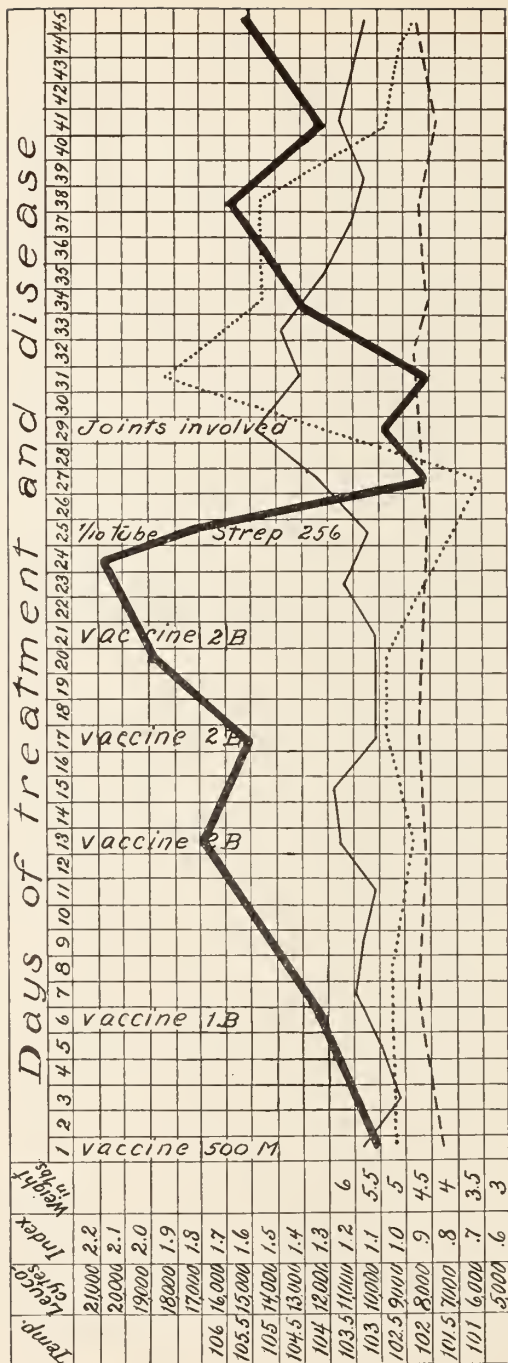


Chart 2.—Prophylactic treatment with vaccine. Rabbit 32. Recovery.
 Heavy solid line—Opsonic index
 Fine solid line—Temperature
 Fine broken line—Weight curve
 Dotted line—Leukocyte count

of the same organism (*Streptococcus* 256). This amount of the living streptococci (256) had been shown by previous experiments to produce an acute arthritis in 95 per cent. of rabbits within four days, the other 5 per cent. dying of septicemia before that time. Suitable control experiments were made in all cases.

The vaccine was prepared in the following manner: The twenty-four hour growth of the streptococcus on blood agar slants was washed off with sterile normal salt solution, the large clumps broken up, and the suspension standardized in the customary way. It was then heated at 60 C. for one hour and tested as to its sterility, enough phenol added to make a 0.5 per cent. solution and the vaccine preserved in the ice chest. Suitable doses were then taken as required. The vaccine was made each week.

The protocols here presented are typical of the experiments and although some differences appeared the essential details were the same in all cases.

Rabbit 26.—On the first, second, fifth, seventh, tenth and twelfth days injected with 500,000,000 to 2,000,000,000 dead streptococci. One-tenth tube of *Streptococcus* 256 injected intravenously on the fifteenth day. On the eighteenth day 2,000,000,000 vaccine given. Found dead with no development of arthritis on nineteenth day.

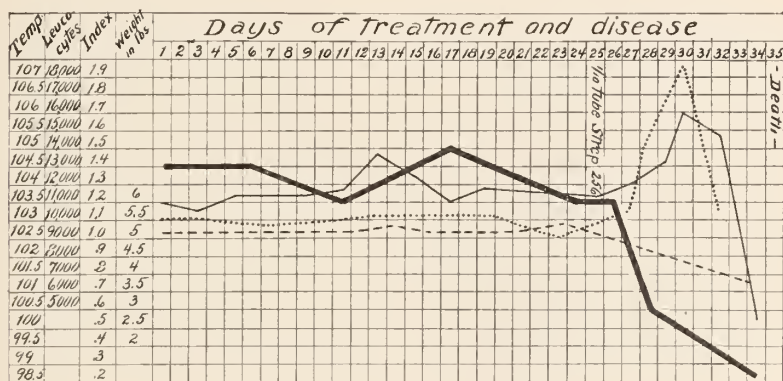


Chart 3.—Control. Rabbit 36.

Heavy solid line—Opsonic index
Fine solid line—Temperature

Fine broken line—Weight curve
Dotted line—Leukocyte count

Rabbit 32.—On the days shown in Chart 2, given 500,000,000 to 2,000,000,000 dead streptococci. One-tenth tube of living *Streptococcus* 256 injected on the twenty-fourth day. Left shoulder joint swollen on the thirty-first day following a dose of 500,000,000 dead streptococci, and metatarso-phalangeal joint of left foot swollen on the thirty-fourth day, following a dose of 1,000,000,000. Rabbit appears well with slightly swollen joints by thirty-ninth day. Three months later rabbit died of epidemic pneumonia. Found left shoulder joint still enlarged.

Rabbit 33.—On the first, fifth, twelfth, sixteenth and nineteenth days given doses of from 500,000,000 to 2,000,000,000 killed streptococci. One-tenth tube of *Streptococcus* 256 injected on the twenty-fourth day. Left wrist swollen on twenty-ninth day. Swelling disappeared by thirty-ninth day. Three months

later rabbit found dead from epidemic pneumonia. All the joints appear normal at necropsy.

Rabbit 27.—Doses of 500,000,000 to 2,000,000,000 streptococcic vaccine given on first, second, fifth, eighth, tenth and twelfth days. One-tenth tube Streptococcus 256 injected on the fifteenth day. Rabbit showed no symptoms of arthritis. On the fifty-second day injected again with 1/10 tube Streptococcus 256. Aborted two days later and died on the fourth day from general septicemia, with no arthritis.

Rabbit 56.—Vaccine administered in amounts ranging from 500,000,000 to 1,000,000,000 on the first, fourth, eighth, twelfth and sixteenth days. One-tenth tube Streptococcus 256 injected on the twentieth day. The rabbit developed no arthritis. Died of epidemic pneumonia two months later.

Rabbit 25: Control: Injected intravenously with 1/10 tube of Streptococcus 256. Left wrist swollen on the third day, right ankle on the fourth day, right elbow on the fifth day. Died on the seventh day.

Rabbit 36.—Control: Injected intravenously with 1/10 tube Streptococcus 256. Both ankles swollen by the fourth day. Death on the ninth day.

Of fifteen rabbits injected with prophylactic injections of vaccine, five, or 33 per cent., were chloroformed on the first, fourth, ninth, tenth and twelfth days, respectively, to complete a series of pathological studies. Of the ten remaining, two died of the acute infection, one on the fourth day, and the other on the ninth day. The other eight, or 53 per cent. of the total number, lived for various periods of from one to six months, an average of three months, and unfortunately died of the epidemic type of rabbit pneumonia, before the duration of the immunity could be determined. If we limit our figures to those which died from natural causes we have eight out of ten, or almost 80 per cent. of recoveries. These figures are significant when compared with the results in the serum and control groups. One, or 6.6 per cent., of the rabbits treated with serum recovered; one, or 9 per cent., of the control animals recovered. A protection is therefore afforded by the vaccine.

Two rabbits were treated with vaccine both before and after the injection of the living organism. Rabbit 26 received a dose of 2,000,000,000 killed bacteria on the third day after injection with the living culture and was found dead on the fourth day, before the appearance of any arthritis. Rabbit 32 received 500,000,000 on the fourth day, before the appearance of any joint involvement. On the morning of the fifth day the left shoulder was swollen. On the seventh and tenth days, vaccine in 1,000,000,000 doses was given with complete recovery by two months. The large dose of dead organisms in the first case may have brought about the rapid death, and in the

second may have caused the joint involvement. However, we cannot make positive statements from these limited data.

In all cases in the serum control groups, the animals had an acute arthritis or died of septicemia, and all gave symptoms of being sick after receiving the living streptococci. On the contrary, two of the vaccine-treated rabbits did not show any symptoms of being sick and had no joint involvement. Rabbit 27 received a second injection of living streptococci (256) one month after the first, and died in three days from abortion and septicemia. Rabbit 28 was injected twice, the injections being forty days apart; after neither inoculation did arthritis or other symptoms appear. While keeping this animal to test the length of the immunity it died from "rabbit pneumonia."

The average number of joints involved per rabbit in each series is as follows: Controls, 2.4; serum treated, 2.6; and vaccine treated, 1.3. The average number of days after the injection of the living streptococcus that joint inflammations appear is: Control, 2.5; serum treated, 3.5; vaccine treated, 4. The latter figures do not include Rabbits 27 and 28 mentioned in the preceding paragraph. Thus the vaccine not only lengthens the interval of incubation but aids in that a milder form of arthritis is produced, and a smaller number of joints is involved.

We employed as a clinical standard of immunity the production of acute arthritis, since this produces symptoms which can be easily recognized in the living animal. A feature equally interesting, which, however, we did not observe until the animals came to necropsy, was the production of endocarditis. Hemorrhages and vegetations on the mitral valves were most common although the tricuspid and aortic valves and the lining of the left ventricle were quite frequently involved. We found endocarditis in 50 per cent. of the controls, 60 per cent. of those treated with serum, and in only 13.3 per cent. of those treated with vaccine. Since eight of the rabbits lived more than one month, it may be said that these may have had an endocarditis and recovered, but of eight⁷ dying within a period of twelve days after receiving the living cocci, only two showed heart lesions. This is 25 per cent., or less than half that shown in the other groups. Of other pathological changes, Dr. Leila Jackson and Dr. E. R. LeCount⁸ found that acute renal lesions occurred less frequently in the rabbits treated with serum and vaccine than in the controls.

7. Rabbit 27, which died 4 days after receiving the second dose of living streptococcus 256, is included in this number.

8. *Tr. Chicago Path. Soc.*, 1914, 11, p. 112.

The reactions produced in the animals by the vaccine and by the injection of living cocci are shown in the charts. The temperature curves show no sharp changes after the vaccine injections, but in all cases there is a rise from 105 to 106 F. after the injection of living cocci. This falls to practically normal within twelve days. The leukocyte count is also variable. It presents nothing distinctive before the live cocci have been injected, after which there is a sharp rise to 18,000 to 22,000; it then recedes and reaches normal by the eighteenth to twentieth day. An important curve is that of the weight, which in the case of the animals injected with vaccine remained stationary, or showed a gradual increase during the entire period. In the cases of the serum and control rabbits, there was a rapid fall in weight in all instances after the introduction of the living cocci, but this did not occur in the vaccine group except in a few rabbits. As seen on the chart, there is but a very slight decrease in weight, which soon returns to the normal.

The opsonins increase during the prophylactic treatment, fall after the live cocci are injected, but soon rise again above the normal. It would seem, therefore, that the immunity is very intimately associated with, and probably dependent on, the production of opsonins.

SUMMARY

Concentrated antistreptococcus serum was prepared according to the methods employed in preparing concentrated diphtheria antitoxin.

Four series of rabbits were injected with this serum: (a) after the establishment of acute arthritis; (b) immediately after receiving live streptococci; (c) before the injection of the live streptococci; and (d) before and again after receiving the living streptococci.

In none of these groups did the concentrated serum appear to have any preventive or curative effect on the acute arthritis.

Experimental streptococcal arthritis was either prevented by, or ran a much milder course after, prophylactic injections of a homologous streptococcus vaccine.

An immunity was produced, which persisted over forty days in at least one case.

The immunity appeared to be intimately associated with, and probably dependent on, the production of opsonins.

Endocarditis and nephritis were less frequent in the vaccine-treated rabbits than in the controls.

THE USE OF DECOLORIZED ACID FUCHSIN AS AN ACID INDICATOR IN CARBOHYDRATE FERMENTATION TESTS WITH SOME REMARKS ON ACID PRODUCTION BY BACTERIA *

W. L. HOLMAN

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In 1895 Andrade-Penny¹ reported his results on the use of "acid fuchsin for the differentiation of bacteria." This author employed an aqueous solution of acid fuchsin (fuchsin S. Grubler) and found it to be an excellent indicator for acids and alkalies. When added to various glycerin media it served to demonstrate the reactions which resulted from the growth and activity of certain of the intestinal bacteria.

In 1906, Andrade² carried the work further, and he strongly advocated the use of glycerin, Dunham's solution, with the acid fuchsin indicator for the differentiation of the bacillus typhosus. For some time our laboratory was employing this medium as a routine for the purpose suggested, and it occurred to us that the indicator could be extended to the other carbohydrates used in our fermentation tests. I therefore added it to various carbohydrate media, and the results proved it to be a very useful addition to our bacteriological technic. I have found it eminently satisfactory as an indicator of the production of acid resulting from the fermentation of carbohydrates by bacteria, and realizing that it is employed in very few bacteriological laboratories, I feel that its use should be more widely extended.

The indicator is prepared as follows:

Acid fuchsin (fuchsin S. Grubler).....	0.5 gm.
Distilled water	100 c.c.

To this solution, which is of rich magenta color, normal sodium hydrate is added until the color is changed to pink, then to a brownish red and this, in turn, is changed to yellow. The change of color takes place slowly, and the solution must be thoroughly shaken after each addition of the alkali. Andrade remarked that a pale pink solution

* Received for publication May 4, 1914.

1. *Ann. Rep. U. S. M.-H. S.*, 1895, p. 385.

2. *Jour. Med. Research*, 1906, 14, p. 551.

will become yellow after standing for an hour or two. It is most practical to add the normal sodium hydrate until a definite yellow color is obtained. It usually takes about 17 c.c. normal soda solution to completely decolorize 100 c.c. of the 0.5 per cent. fuchsin solution. One c.c. of this indicator is added to 100 c.c. of the carbohydrate medium.

I have found that broth made \pm 0.6 to phenolphthalein (hot titration) remains colorless on the addition of the fuchsin indicator prepared as described above. During sterilization at 100 C. the broth turns a distinct pink. This color disappears on cooling, and the medium remains colorless at room and incubator temperature.

Undoubtedly the acid in the broth is partially neutralized by the addition of the indicator, as the final broth is found to be neutral to phenolphthalein in the cold. I have found as has Winslow³ and others that the use of phenolphthalein at 100 C. shows slightly higher acid reaction than when used in the cold. This gives a double control on a neutral point for the broth; a slight reaction in the cold to phenolphthalein but not to the fuchsin indicator, and at 100 C. a slight acid reaction to the fuchsin but none to phenolphthalein.

The indicator is made up in large quantities. It should be tested twenty-four hours after the last addition of the alkali by adding it to \pm 0.6 broth in 1 per cent. quantities. A pink color appears on boiling and this on cooling fades, leaving the broth unchanged in color.

This indicator is very sensitive to the presence of the organic and mineral acids. One thousand c.c. of water containing 1 drop of concentrated lactic acid, butyric acid, acetic acid, or tannic acid (20 per cent. solution) turns pink on the addition of the indicator.

During the fermentation of the various carbohydrates by bacterial growth, organic acids (most frequently lactic acid) are produced, any one of which, acting on the fuchsin salt, sets free the fuchsin, and turns our media pink or red. The depth of the color depends roughly on the amount of acid produced.

The fuchsin salt is very stable. It is not affected by heating to 100 C. and over, and no dissociation takes place after the indicator has remained for years in the media. It has no appreciable effect on the growth of even the most sensitive organisms.

Mereshkowsky⁴ says that fuchsin in a dilution of 1-1,000 delays, to some extent, the action of invertase, and occasionally renders it

3. Systematic Relationships of the Coccaceae, New York, 1908.

4. Quoted by Fuhrmann, Bakterienenzyme, Jena, 1907.

inactive. The fuchsin, as used in the Andrade indicator, is in a dilution of over 1-20,000 and in our experience has no demonstrable effect in checking or delaying the action of this ferment in the fermentation of saccharose. I have made a number of comparisons to definitely determine this point, using azolitmin and Andrade's fuchsin as indicators. A quantity of saccharose broth was divided into equal parts, the two indicators added, tubed in Durham's fermentation tubes, and sterilized together. A number of strains of the bacilli proteus, cloacae, coli communior, lactis aërogenes, acidi lactici, xerosis, together with yeast were used to seed the medium. The results showed: (1) that acid was indicated earlier in the tubes containing Andrade's indicator; (2) that no acid was shown by either indicator in the tubes seeded with non-saccharose fermenting bacteria; (3) that the amount of gas formed by the acid and gas fermenters was the same; and (4) that the litmus was rapidly decolorized by many of the strains making the reading for acid difficult or impossible. From these results and experience extending over several years, I conclude that Andrade's indicator has decided advantages over litmus or azolitmin in that: the indication of the presence of acid is clear-cut and definite; the cultures may be examined and the reading made as well by artificial light as by daylight; it is unnecessary to make comparisons with a control tube; as our media in the neutral state is colorless, there is not the difficulty of making every batch of media exactly the same shade of violet as with litmus; litmus is also much more easily decolorized by reduction, which adds to the difficulty of determining acid production.

For teaching purposes and for use by the student, Andrade's indicator fills a long-felt want.

The method of titrating with phenolphthalein, in order to obtain the exact percentage of acid produced after different periods of growth, is too cumbersome for routine laboratory work and offers little or no help in the diagnosing of our cultures. In the differentiation of bovine from human strains of the bacillus tuberculosis, however, the titration determinations are of great importance as first shown by Theobald Smith. There are, however, too many variable factors to be considered to make it of practical use excepting in these very special biological researches. The number of organisms transferred, the age of the culture used for the planting, and the late previous environment of the strain, all lead to wide differences in the exact percentage of acid pro-

duced in a given time by one and the same organism. It is often difficult, when one makes transfers of one organism to a number of different tubes or flasks of even the same lot of medium, to obtain the same titration results from the whole series. Working with different lots of media, however carefully made, the difficulty of obtaining identical results is increased.

Andrade's indicator is useful in making titration determinations of the acid produced, where this is particularly desired. Measured quantities of alkali are added to known amounts of media in test tubes or flasks until the pink color disappears. Precaution must be taken to allow sufficient time for the adjustment between the acid and alkali to take place.

The presence of slight amounts of alkali in carbohydrate media does not interfere with the indication of acid production as so many investigators seem to believe. Unless the alkali is present in such quantities as actually to prevent the growth of the organisms, the acid soon neutralizes the alkali and growth and fermentation continues until the acid produced is sufficient to either kill the bacteria or to interfere with their specific biological functions. Winslow in a study of thirty-three strains of cocci concludes that an excess of acid over 1 per cent. is more generally fatal than an alkaline reaction. The presence of acid in media has a very detrimental effect on the life of many bacteria, such as the cholera vibrio, certain streptococci, and many others.⁵ The addition of carbohydrates to media increases the growth of bacteria, which have the power to ferment the particular carbohydrate, but lessens the longevity of the organisms.

The acid produced in fermentation first affects the biological function of producing ferments, or lessens the action of such ferments if produced, and then the acid gradually kills the bacteria. It is true, however, that very rarely ferments have been known to outlive the bacteria.⁶ Fuhrmann⁷ refers to the large number of bacteria which break up the simple sugars to lactic acid, and says that these bacteria are very sensitive to the action of acids, especially lactic acid, which checks their growth and activity.

The fermentation of a given carbohydrate by an organism is a definite biological character of that organism. The acid death point is a question of vital resistance. The acid point at which bacterial

5. Lafar, *Handb. d. Tech. Mykol.*, Jena, 1907; *Am. Jour. Pub. Health*, 1913, 3, p. 1210.

6. Lafar, *Handb. d. Tech. Mykol.*, Jena, 1907.

7. *Bakterienenzyme*, Jena, 1907.

functions cease is a question of biological functional resistance. I have shown that by adding sterile sodium hydrate solution (N/20) to dextrose broth cultures of streptococci each day, to prevent the accumulation of acid, the total acid produced is increased three to four times over that in the unneutralized controls. Similar methods were used by Fischer⁸ in determining the acid production of the bacilli coli and paratyphosus.

The determination of the acid death point and the point at which biological activities cease, or are depressed, should be distinguished from the determination of the qualitative fermentative powers of bacteria.

Biometrical studies of fermentation powers of bacteria, in which an arbitrary acid point is spoken of as fermentation while all acid production below this point is neglected, are of great interest as indicating the variable sensitiveness of different bacteria and their ferments to the acids produced, though of little use in determining whether the organism has or has not the power to reduce the carbohydrates. Winslow⁹ for streptococci fixed the fermentation point at +1.2 to phenolphthalein, while Broadhurst uses +1.5. Streptococci showing acid reactions at or below these points are spoken of as non-fermenters. A reaction of +0.8 per cent. turns litmus a decided red.¹⁰ What is the source of their +1.2 or +1.5 per cent.? I believe it to be the result of the fermentation of the contained carbohydrate. The biometrical system of classification by fermentation tests of Andrewes and Horder¹¹ and the other English workers is based on qualitative changes. It is true, that subdivisions might be made by the use of further quantitative determinations, but much more must first be learned of the sensitiveness of the bacteria and the conditions of the activity of their enzymes before such a classification is, we believe, advisable. We have found as have many investigators that strains of the bacillus coli recently isolated from water show marked depression in their fermentative powers. Henningsson¹² has shown that cultures of the bacillus coli kept in water for long periods will suffer quantitative loss in their fermentative powers. These depressions are not, however, considered worthy of use in classification:

9. Systematic Relationships of the Coccaceae, New York, 1908.

8. *Centralbl. f. Bakteriöl.*, 1911, 59, p. 474.

10. *Ibid.*

11. *Lancet*, 1908, 171, p. 708.

12. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1913, 74, p. 253.

It is well known that different carbohydrates broken up by fermentation give different reduction products. Lactic acid is most constantly found, but oxalic, butyric, acetic, succinic, formic, and other acids are also formed.¹³ Moreover, different bacteria break up the same carbohydrate with varying final products.

The fact that bacteria are destroyed by the acids produced in fermentation is well established. It also appears that certain ferments formed by the bacteria are inactive in acid media, or are not formed under the unfavorable acid environment, because the amount of acid produced by the same organism on different carbohydrates varies. Winslow,¹⁴ Fuller and Armstrong,¹⁴ and others, have shown that the amount of acid produced by streptococci from the reduction of dextrose is much greater than that from the more complex carbohydrates, such as lactose, mannite and raffinose. Broadhurst¹⁵ shows that the percentage of acid from salicin is higher than from these three carbohydrates.

The fact that acid is produced by these organisms from the higher carbohydrates, even though the amount is less than from the lower forms, indicates that the power of reducing these carbohydrates is present, and that fermentation does not continue must be due to the presence of the acid products and the lowering of the specific functional activity of the organism or the ferment. Bacterial invertase, for example, is sensitive to acid. Emulsin, however, is not affected by the organic acids (Fuhrmann).¹⁶

We have used this neutralized acid fuchsin indicator in studying the fermentation of the various carbohydrates; dextrose, maltose, lactose, saccharose, dulcitol, raffinose, salicin, mannite, glycerin, dextrin, and others, with a large number of different bacteria including the pneumococcus, streptococcus, the colon-typhoid, and the diphtheria groups, the Gram-negative cocci, and many others, and have found it very satisfactory. The secondary alkali production, among certain of the bacteria, is definitely shown by the decolorization of the indicator. This decolorization is, however, at times, due to the reducing power of the bacteria on the fuchsin. We have noted it particularly among members of the *Bacillus mucosus capsulatus* group. The decoloriza-

13. Gotschlich, Kolle and Wassermann, *Handb. d. path. Mikorg.*, Jena, 1913; Fuhrmann, *Bakterienenzyme*, Jena, 1907; Lafar, *Handb. d. Tech. Mykol.*, Jena, 1907.

14. Systematic Relationships of the *Coccaceae*, New York, 1908; *Jour. Infect. Dis.*, 1913, 13, p. 442.

15. *Jour. Infect. Dis.*, 1912, 10, p. 272.

16. *Bakterienenzyme*, Jena, 1907.

tion, due to the reducing power of the bacteria, appears much less often and at a far later period of growth than when litmus is used.

CONCLUSIONS

The neutralized acid fuchsin indicator as used by Andrade in his glycerin media is applicable for testing the production of acid in the bacterial fermentation of all carbohydrates.

The technic of obtaining a neutral point in media by hot titration with phenolphthalein controlled by the acid fuchsin indicator, showing pink when hot and colorless when cold, gives the most satisfactory results.

Andrade's neutralized acid fuchsin is superior to litmus, as an indicator of acid production in media, on account of its sensitiveness, the sharpness of the change of color (Cohn),¹⁷ and the higher resistance to reduction decolorization. It is particularly useful for teaching purposes.

Titration determinations can be carried out with this indicator at any stage in the growth of the culture.

The use of this indicator in all bacteriological laboratories would give better and more comparable results in studies on the fermentation of the carbohydrates.

Qualitative tests for fermentation are more important than quantitative in the classification of bacteria.

The resistance of bacteria and their ferments to the effects of acid produced in the fermentation process differs with the carbohydrate reduced and with various strains of the same organism. These differences are not, we believe, sufficiently understood to warrant their use in classification.

17. Indicators and Test Papers, New York, 1910, p. 2.

EXPERIMENTS ON THE VARIABILITY OF THE FERMENTATIVE REACTION OF BACTERIA, ESPECIALLY THE STREPTOCOCCI *

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Within the past few years certain chemical substances, especially the carbohydrates, have been used in the separation of species of bacteria. It is in the separation of the members of the typhoid-colon group and the Gram-negative cocci¹ that these substances have been of especial service. These chemicals have also been used by a number of investigators in separating the streptococci into groups. Further, some of them have considered that the fermentation tests might be of practical value in determining the types of streptococci concerned in water pollution.

After trying only a few strains of streptococci on some of these fermentable substances, I was impressed by the great variability of the results, and on reading the literature of the subject I found that previous investigators of the same group of bacteria did not agree. It seemed to me that it would be profitable, after establishing the fact that there was variability, to try to discover the causes.

By perusal of extracts from articles of my predecessors, one can readily see that their results are not in entire accord, and that some of them are very skeptical as to the value of the carbohydrates in separating streptococci. Out of 300 streptococci from saliva, Gordon² found that there were no less than 48 types, all of which fermented saccharose, and none fermented mannite. He gives this conclusion after trying out strains pathogenic for man. "These and the other results recorded in the table indicate that there is considerable diversity amongst streptococci occurring in sepsis."

Houston³ examined 300 strains from feces using eight of Gordon's tests, and found forty types.

Andrewes and Horder⁴ believe that if Gordon had used twenty tests instead of nine that instead of forty he would have found one hundred or more varieties amongst them.

Buerger⁵ tested thirty-four strains of streptococci using sugar-free broth or beef-serum and water and found six varieties with the carbohydrates among only thirty-three strains.

* Received for publication November 15, 1913.

1. *Jour. Med. Research*, 1909, 20, p. 369.

2. *Lancet*, 1905, 2, p. 1400.

3. *Suppl. Ann. Rept.*, 1904, p. 326.

4. *Lancet*, 1902, 2, p. 708, 775, 852.

5. *Jour. Exper. Med.*, 1907, 19, p. 428.

Winslow and Palmer⁶ investigated 302 cultures from feces. Comparing their results with those of Andrewes and Horder they state, "The various investigations are concordant with the exception that rhamnose-fermenters in both human and bovine feces were less frequent than in those of their predecessors."

E. W. Ainley Walker says: "The reactions for the streptococci exhibit an extraordinary degree of variability if observed over extended periods of time, or after changes in their environment likely to encourage the appearance of variations. It follows that the streptococci cannot be classified into varieties and sub-varieties on the results of their reactions in sugar-containing media."

Libman and Celler⁸ found that inulin as a substance to differentiate between streptococci and pneumococci is not constant, as out of sixty-nine streptococci, two fermented inulin, and of the nineteen pneumococci, two did not.

Beattie and Yates⁹ state: "In our hands Gordon's tests have proved quite unreliable in differentiating strains of streptococci."

Gordon¹⁰ in his reply to Andrewes and Horder's criticism states that Twort has shown that the bacillus typhosis can be coaxed into fermenting lactose. He says, "I will not admit that streptococci are extraordinarily variable in their reactions."

Bergey¹¹ concludes that the study of the streptococci, for the purpose of differentiating cultures, derived from different sources, has strongly emphasized the unsatisfactory nature of the method of differentiation through carbohydrate fermentation.

Broadhurst¹² used six fermentable substances and one hundred strains of streptococci (from milk). They fell into twenty groups.

Stowell, Hilliard and Schlesinger¹³ found that a comparison of their work with that of previous workers does not show any great uniformity in results. In regard to the stability of the fermentation tests they conclude that reinoculation after a week or longer usually confirmed the first findings.

In my work the strains of streptococci isolated from different sources were tried on the following chemicals: dextrin, arabinose, mannite, salicin, raffinose, lactose, saccharose and inulin. Dextrose, galactose, dulcitol, maltose and levulose were not used to any great extent as they are not of much use in differentiating streptococci. Agar, containing sugar-free broth from fresh meat and made neutral to Kubel Tiebmann's litmus, was used. The carbohydrates (Merck's or Kahlbaum's) were sterilized separately for only fifteen minutes in the Arnold sterilizer and added to the melted agar, which was then incubated at 37 C. for two days. The contaminated tubes were then discarded. All the strains were tried in blood agar and by this means were separated into three groups, that is, those producing green pigment, those producing hemolysis, and those producing pneumococci.

6. *Jour. Infect. Dis.*, 1910, 7, p. 1.

7. *Jour. Path. and Bacteriol.*, 1911, 15, p. 124.

8. *Am. Jour. Med. Sc.*, 1910, p. 140, 516, 527.

9. *Jour. Path. and Bacteriol.*, 1911, 16, p. 247, 137.

10. *Ibid.*, 1911, 15, p. 323.

11. *Jour. Med. Research*, 1912, 27, p. 67.

12. *Jour. Infect. Dis.*, 1912, 10, p. 272.

13. *Ibid.*, 1913, 12, p. 144.

All were considered pneumococci that had well-developed capsules, that fermented inulin, that were green in blood-agar, and that were dissolved by bile. The results of the fermentation tests are given in the following tables.

Out of six strains of streptococcus hemolyticus only three were alike, and they were isolated from the same original culture.

TABLE 1
STREPTOCOCCUS HEMOLYTICUS

No.	Blood Agar	Source	Dextrin	Arabinose	Mannite	Salicin	Raffinose	Lactose	Saccharose	Inulin
3	Hemolysis	Blood (septicemia)	+	—	+	+	+	+	+	—
4	Hemolysis	Blood (septicemia)	+	—	+	+	+	+	+	—
18*	Hemolysis	Tonsil (acute tonsillitis)	+	+	+	+	+	+	+	—
19	Hemolysis	Tonsil (chronic arthritis)	+	±	—	—	±	+	+	—
20	Hemolysis									
27	Hemolysis									

* 18, 19, 20, different colonies from one culture.
+ = acid formed; — = neutral; ± = doubtful.

TABLE 2
PNEUMOCOCCUS

No.	Blood Agar	Source	Dextrin	Arabinose	Mannite	Salicin	Raffinose	Lactose	Saccharose	Inulin
11	Green	Sputum (Pneumonia)	++	—	—	++	+	+	++	+
23	Green	Cerebrospinal Fluid	++	++	++	++	+	+	++	+
25	Green	Sputum (Pneumonia)	++	—	++	++	—	++	++	++
32	Green	Blood Culture (Pneumonia)	+	—	—	—	+	+	++	+
35	Green	Pneumonia Cerebrospinal Fluid	+	+	—	—	+	+	+	+

+ = acid formed; — = neutral.

Of the five strains of pneumococci no two strains were alike.

Table 3 includes the streptococci that produced green in blood-agar, and which we call the streptococcus viridans. The green-producing cocci that peptonize milk are classed as the streptococcus zymogenes.¹⁴

Table 3 shows the behavior of the streptococcus viridans on arabinose, dextrin, salicin, raffinose, mannite agar, unless otherwise indicated. For example, Strains 13, 14 and 28 fermented all five carbohydrates.

A study of these results, and a comparison of them with those of previous workers will lead one to conclude that there is a remarkable variation in the fermentative reactions of the streptococci.

TABLE 3
STREPTOCOCCUS VIRIDANS

No.	Source					
13	Blood culture (endocarditis)					
14	Tooth	+	+	+	+	+
28	Tonsil					
7	<i>M. rheumaticus</i> B.					
12	Blood culture (endocarditis)					
15	Tooth	+	+	+	+	Raffinose —
16	Eye					
17	Infected wound					
26	Skin					
5	Urine					
6	Anus	+	+	+	+	Arabinose —
8	Tonsil					
21	Blood culture (endocarditis)					
29	Tooth	+	+	+	+	Mannite —
31	Blood culture (endocarditis)	+	+	+	Raffinose —	Mannite —
34	Tooth	+	+	+	Salicin —	Mannite —
30	Tooth	+	+	Dextrin —	Raffinose —	Mannite —
10	Tooth	+	+	Arabinose —	Raffinose —	Mannite —
33	Tooth	+	+	Salicin —	Raffinose —	Mannite —
24	Joint	+	Arabinose —	Salicin —	Raffinose —	Mannite —
9	Tooth	+	Arabinose —	Dextrin —	Raffinose —	Mannite —
1	Blood culture (endocarditis)	+	Arabinose —	Dextrin —	Raffinose —	Mannite —
2	Blood culture (endocarditis)	+	Arabinose —	Dextrin —	Raffinose —	Mannite —
22	Cerebrospinal fluid	—	Arabinose —	Dextrin	Raffinose —	Mannite —

The problem now seems to be to determine, if possible, the cause of these great variations.

One colony of Strain 12, obtained from the blood in a case of infectious endocarditis, was plated and ten colonies were transplanted to agar tubes.

The ten colonies were transplanted to sugar-free lactose broth and after determining that the growth was pure, phenolphthalein was

TABLE 4
ACID REACTION OF TEN COLONIES FROM STRAIN 12

No. of Colonies	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
1	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
2	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
3	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
4	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
5	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
6	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
7	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
8	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
9	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+
10	N	+	+	+	+	+	N	+	+	+	+	+	+	+	+	+	+	+

N = neutral; + = faintly acid; ++ = distinctly acid; +++ = very acid.
The figures I-XVIII refer to the different transplants.

added and the broth was titrated, but no increase in acidity was detected. Later, these colonies would not grow in the sugar-free broth containing lactose nor in the same added to agar.

The ten colonies were transplanted to dextrin sugar-free agar, and the results are in Table 4.

Even in the first trial there was variation of the colonies, since in six of the tubes the reaction was acid, while in the remainder no fermentation took place.

On the third trial Colony 7 produced such a large amount of acid that it suggested contamination, but on plating it out, it was found to be pure.

On the fifth and sixth trials the dextrin was filtered through a Berkefeld filter and not heated, but there was still variation in the amount of acidity.

That the variation was not due to lack of uniformity in the titration or in the dextrin, is proved by the great discrepancy between the eighth and ninth trials, which were made on the same lot of agar, and between the tenth, eleventh and twelfth trials, which likewise were grown on the same lot of agar.

With successive inoculations on dextrin it was found that, as a rule, the power to produce acid was increased. The exceptions to this rule, Colonies 7 and 8, which formed very little acid in the last trials, died out soon after the trials were made.

In the thirteenth trial, Colonies 1 and 10, only, were in ordinary glass and there was very little acidity, but there was likewise very little acidity in 7 and 8, grown in the quartz tubes.

In the fourteenth trial Colonies 1 and 9, only, were in ordinary glass and there was just as much acidity as the most produced in quartz glass, also there were great variations in the amount of acidity produced by the different colonies in quartz tubes. In Colony 10, which was very faintly acid, the growth was scanty. It might seem that the amount of growth is an important factor, but there were instances in which the growth was alike with great variations in acidity.

In the fifteenth trial, Colony 7, in a quartz tube, did not produce any acid at all, and Colony 10 produced only a slight amount.

In the sixteenth trial, Colonies 3, 7 and 8, grown in the quartz tubes, produced less acid than the other colonies.

Colony 1 was grown in both ordinary and in quartz tubes, and both were very acid. The agar was decolorized near the bottom in the ordinary glass tube; but in this tube there was a greater distance from the bottom of the tube to the surface of the agar. In other instances, however, the depth of the agar did not influence the decolorization.

In one case where the same colony was grown in both ordinary and in quartz glass there was less acid produced in the ordinary glass.

In the eighteenth trial the cultures were grown under anaerobic conditions, and Colonies 7 and 8 produced less acid than the others. After this trial Colonies 7 and 8 died.

It was noted in the sixteenth trial that one colony grown in the quartz tube was not so acid as the same colony grown in the ordinary glass and that the latter had water of condensation present. In order to learn if the water affected the result, the same colony was simultaneously inoculated on tubes of media from which the water of condensation had evaporated, and on some of these tubes that had distilled water added to them after which they were sealed. In the latter case more acid was produced. The water may have aided the dissemination of the acid through the media.

The ten colonies of Strain 12 were planted in nutrose water medium plus 1 per cent. of dextrin. The medium had been previously incubated, and found sterile. All the colonies grew well in this medium. At the first trial there were marked differences varying from neutral, in two colonies, to marked acidity. This same medium was placed in quartz test tubes and the two colonies mentioned above remained neutral.

In the following experiments the ten colonies were obtained from one colony.

Ten colonies of Strain 30 were planted twice on raffinose agar without any variations appearing, since none of the colonies fermented this substance.

Ten colonies of Strain 28 were planted twice on raffinose agar and on the second trial there was a slight variation. Some did not ferment at all while others produced a slight acidity.

Ten colonies of Strain 36 were planted on raffinose agar. The medium was freshly made and the water of condensation was present. There was a good growth in every tube. At the first trial all were neutral except Colonies 8 and 10, which were strongly acid. At the next trial, on the same lot of media, similar results were obtained, but Colony 8 was not so distinctly acid. The ten colonies were then

placed on mannite agar and all were neutral except 8 and 10, which were moderately acid.

Ten colonies of Strain 37 were planted on raffinose agar and at the very first trial there was marked variation which ranged from neutral to marked acidity. Colonies 1, 2, 6, 9 and 10 were neutral 3 and 8 were faintly acid, and the remainder were markedly acid. When these ten were planted on mannite they all produced a large amount of acid. Ten colonies derived from one colony of Strain 13 were placed on arabinose for four successive times. On the first trial two did not ferment and the remainder were doubtful. On the second trial Colony 10 produced more acid than the remainder. On the third all but Colony 4 produced acid. On the fourth trial all the colonies fermented the sugar extensively.

In the experiments with the *micrococcus rheumaticus*, ten colonies were obtained as follows: a streak culture was made on agar in a petri dish and one colony was fished off and a streak culture made on each of two petri dishes, and the ten colonies were then fished from the second dish.

The first trial was made on raffinose in sugar-free litmus agar tubes that were old. There was no water of condensation present. None produced acid.

The second trial was made on fresh agar containing raffinose. All were neutral except Colony 5, which changed the entire amount of agar to a distinct red. Similar results were obtained on the third and fourth trials.

Colony 5 was streaked on agar in a petri dish and the five colonies fished off distinctly acidified raffinose, while five colonies from Tube 4, which was neutral, did not acidify raffinose at all. The ten original colonies were planted on arabinose and all (except Colony 5, which remained neutral) produced a large amount of acid.

These two distinct colonies were plated in blood-agar and found to be green. The growth was also similar in broth and in dextrin litmus agar. In mannite litmus agar, one produced more acidity than the other.

Therefore it seems that there were two distinct strains in this culture, or else we have here another instance of variation.

The result of experiments with the *micrococcus rheumaticus* are given in Figure 1.

I compared my results with those obtained by Rosenow,¹⁵ using the same culture of the micrococcus rheumaticus, and found that we disagreed in our results in the cases of three carbohydrates: raffinose, inulin and mannite.

Miss Jean Broadhurst (using the titration method) tried out three of my strains and her results agreed with mine.

The behavior of the colonies of Strain 12 on lactose is interesting. When first isolated they grew luxuriantly in the lactose sugar-free broth, but after a number of transplants on dextrin, they were placed on lactose agar made of sugar-free broth. Only half of the colonies grew, and the fermentation of lactose was irregular. This experiment was repeated several times with similar results. Recently the colonies were planted in sugar-free broth containing lactose, but none grew. On repetition, the same result was reached. Other strep-

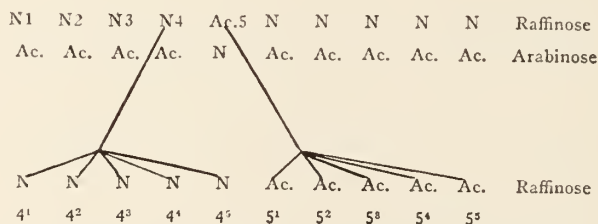


Fig. 1.—Result of Experiments with the micrococcus rheumaticus.

tococci grew in this broth luxuriantly. Thinking that the colonies could be brought to grow on the sugar-free agar they were transplanted to litmus milk. There was abundant growth. They were then transplanted to broth, not sugar-free, where there was good growth. They were then put back on the lactose agar made of sugar-free broth but there was no growth whatever. It seems that as the colonies began to die out their power to grow in a sugar which they did not ferment became diminished.

Six colonies fished from the original plate, made at the time of the removal of a piece of the tumor from the nose in a case of rhinoscleroma, gave variable results. Colonies 1 and 2 fermented lactose, while the remainder did not. Colonies 1, 2 and 3 fermented saccharose, while the remainder did not. This cannot be due to scanty growth, since the growing powers of this organism are well known.

15. *Jour. Infect. Dis.*, 1910, 7, p. 413.

In order to learn whether long cultivation of organisms on artificial media changed their fermentation power, two strains of *Bacillus mucosus*, which had been kept alive for nearly three years, were tried out on the same carbohydrates. In one strain dulcitol had not been fermented, but it did produce acid on the second trial. With the other strain the power to ferment dextrin was lost. A strain of *Bacillus rhinoscleromatis*, which had been grown on artificial media for three years, had lost the power to ferment dextrin.

CONCLUSIONS

The variability in the results of the action of bacteria on certain chemical substances may be due to:

The use of media that have become dry and lost the water of condensation.

Faulty preparation of media: not exhausting the meat sugar from the broth, or overheating the carbohydrate.

The variability in the growing powers of different strains of streptococci and pneumococci.

Length of time grown on artificial media.

The complexity of the substances used and the difficulty of obtaining absolutely pure chemicals: dextrin, arabinose, etc. Different investigators have not used the same samples of carbohydrates.

The human element which enters into the titration of the media, and it is probable that the reaction varies with different workers.

A small amount of alkali from glass tubes which would be sufficient to change a faint acid reaction to a neutral.

The exceptional variability of the group of bacteria. It seems that some of these experiments prove this to be true.

Some of the disadvantages are obviated by the use of a liquid medium instead of agar. But if liquid media are used the results are still modified by the alkali in the glass, overheating, complexity and variability of the chemicals, variability of the growth, and the human element in the titration.

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TUBERCULOCIDAL ACTION OF CERTAIN CHEMICAL DISINFECTANTS*

STUDIES OF THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS, IX

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In all the great mass of literature on general disinfection, we have been able to find but little dealing at all directly with the power of chemicals to kill tubercle bacilli. It has been generally accepted that tubercle bacilli, though non-sporogenous, are among the most resistant of pathogenic organisms.

Drying-out processes seem to have little effect on either their vitality or their pathogenicity. Authors have differed regarding the action of dry heat, but Krumwiede,¹ in carefully controlled experiments, found that tubercle bacilli dried for five days and subjected to dry heat at 100 C. were killed in one hour and fifteen minutes so that inoculated animals failed to develop the disease. Some tubes were killed in twenty minutes. Theobald Smith² noted that cultures of tubercle bacilli, which had been kept from six weeks to two months, would not grow when transferred to fresh tubes of glycerin agar. Supposing such cultures to be dead, he used them for immunization experiments on guinea-pigs and found that cultures, which had been kept from seven to twenty months,

* Received for publication May 11, 1914.

1. *Rept. Research Lab. Dept. of Health*, New York City, 1913.

2. *Jour. Med. Research*, 1913, 28, p. 91.

still contained a sufficient number of living bacilli to confer the disease upon susceptible animals. The earliest report found of chemical disinfection of tubercle bacilli was published in 1888 by Yersin.³ Earlier experiments on the disinfection of tuberculous sputum had been published, but as these do not permit of scientific accuracy and control, they will be omitted and we will review only those dealing with the action of chemicals on pure cultures of tubercle bacilli. Yersin used pure cultures fifteen days old. He exposed small bits to the action of his disinfectants for variable times, then dropped the bits into distilled water. After several hours in the sterile distilled water, the well-washed bit of culture was transferred to flasks of glycerin broth and incubated at 39 C. If no growth developed, he concluded that the organisms were killed. Thus he states that tubercle bacilli exposed for 30 seconds to 5 percent phenol, or one minute to 1 percent, were killed. Absolute alcohol killed in five minutes, iodoformed ether, 1 percent, killed in 5 minutes, while pure ether required 10 minutes, and 0.3 percent thymol destroyed the organisms in two hours. This report is interesting historically, since it is one of the earliest disinfectant reports. It is also interesting because it is the only investigation which we have found in the literature in which any systematic study of the action of chemical disinfectants on tubercle bacilli has been made. It is unfortunate that the author confined his tests, on the death of the organism, entirely to artificial cultures, and inoculated no animals with the treated bacilli, since, as Theobald Smith stated, the animal body is the best culture media for the growth of tubercle bacilli. It is also unfortunate that he did not realize that while the organisms were being washed in distilled water, they were under the influence of the diluted disinfectant, since, if we understand him correctly, the water was not changed, nor was any effort made to neutralize the disinfectant. It can scarcely be doubted, therefore, in the light of more recent disinfectant work, that the times reported in his experiments must inevitably be too short.† Practically all the other reports found by us have been either on the disinfection of sputum and other excreta or brief mention of tubercle bacilli used with many other bacteria in the larger works on the testing of disinfectants. Thus Bechhold,⁴ in testing halogen derivatives of phenol and naphthol groups, found that they had a much stronger action than lysol on the staphylococcus aureus, streptococcus, the bacilli coli, typhosus, diphtheriae, etc. On human tubercle bacilli, however, they had no effect; a 2.5 percent solution of tri-tetra-brom β Naphthol failed to kill these organisms in twenty-five hours, although 5 percent lysol containing 2.5 percent cresol killed the tubercle bacilli in four and one-half to eight hours. In Bechhold's experiments, emulsions of human tubercle bacilli were exposed to equal volumes of the disinfectant, and then injected into animals. No attempt was made to remove the disinfectant or to check its action, as the disinfectant and organisms were injected together. Inhibition may therefore play some part, although the long time required for the disinfectant action of lysol suggests that its part cannot have been very great. Green⁵ found that chloroform vapor mixed with air and passed through tubes containing vaccine lymph and different kinds of bacteria, including tubercle bacilli, sterilized the lymph so that no growth developed when it was inoculated

3. *Ann. de l'Inst. Pasteur*, 1888, 2, p. 60.

† At the time at which Yersin worked slight attention was paid to the strain of tubercle bacillus used. Discrepancies between the results of the French and the German workers led to an investigation of this point, and it was found that the French workers were dealing with bovine, while the German bacteriologists were studying human, tubercle bacilli. So Yersin's work was done with pure cultures of tubercle bacilli, but they were undoubtedly of the bovine type.

4. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1909, 64, p. 113.

5. *Lancet*, 1904, 1, p. 1498.

on suitable media. Cantani⁶ states that iodine in dilutions of 1 to 500, or even 1 to 1,000, had considerable influence on tubercle bacilli. Benians⁷ found that emulsions of tubercle bacilli shaken for fifty minutes, or for twenty-four hours with 10 percent toluol, when injected into guinea-pigs caused no infection. Wells and Corper⁸ in a similar way found that tubercle bacilli exposed for twenty hours, and for five days to toluol water, and then injected into guinea-pigs, in no case caused tuberculosis. Lumière and Chevrotier⁹ investigated a large number of metallic salts with reference to their inhibitory action on tubercle bacilli. Cadmium chlorid and mercuric chlorid were the most active in inhibiting growth, 1 in 4,000 being sufficient for that purpose. Rosenthal¹⁰ finds that gold cyanid in dilution of 1 to 2,000,000 inhibits the growth of tubercle bacilli, but he admits that the action is inhibitory, as he obtains a luxuriant growth if he washes the organisms after seventy-two hours' exposure to dilute gold cyanid solutions. Biasiotti¹¹ states that colloidal silver in high dilutions kills the staphylococcus aureus, the bacillus typhosus and diphtheriae, and, undiluted, kills the spores of the bacillus anthracis, but not tubercle bacilli. May¹² found that basic fuchsin had some germicidal action, killing the bacilli typhosus, paratyphosus, coli, and dysenteriae in five minutes. He also found that the staphylococcus aureus and the saprophytic form of the tubercle bacillus could be killed by this dye. DeWitt¹³ has shown that methylene blue, in high dilution, inhibits the growth of tubercle bacilli, and while not all the organisms are killed, the vitality of the culture is so lowered that the disease develops slowly, frequently causing a local process only. Mercury salts or trypan-blue also showed marked tuberculocidal properties, only one out of the six animals used developing the disease.

Since, then, so little has been systematically done on the chemical disinfection of the tubercle bacillus, it has seemed advisable to test a number of the common disinfectants in order to determine their action on human tubercle bacilli. It is probable that the small amount of work on this problem is due less to lack of appreciation of its importance than to the difficulties that one encounters in finding a method, which is at the same time accurate and not too time-consuming and complicated.

At the beginning of our investigations, we tried numerous methods—the silk-thread method of Koch, the garnet method of Krönig and Paul, and emulsion methods. We early found that no emulsion method could be depended on to give uniform results on culture tubes. Some tubes would show growth, which was usually delayed, but the breaking up of clumps and separation of the organisms from each other seemed sufficient to prevent or greatly delay growth on agar tubes

6. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1909, 63, p. 34.

7. *Ztschr. f. Chemoth.*, 1913, Orig., 11, p. 28.

8. *Jour. Infect. Dis.*, 1912, 11, p. 388.

9. *Bull. gén. de therap.*, 1913, 165, p. 959.

10. *Ibid.*, p. 961.

11. *Ann. d'ig. sper.*, 1909, 19, p. 543; Reviewed in *Centralbl. f. Bakteriöl.*, 1910, Ref., 45, p. 680.

12. *Jour. Am. Med. Assn.*, 1912, 48, p. 1174.

13. *Jour. Infect. Dis.*, 1913, 12, p. 68, and 13, p. 378.

or even in glycerin broth, even without exposure to any disinfectant. In other words, single organisms, separated from their companions, lack the vitality necessary for uniformly successful culture on artificial media. It seemed necessary, therefore, to expose a clump of the bacteria to the action of the disinfectant. The time of exposure in nearly all experiments was one hour, six hours, and twenty-four hours. As we desired to control the time sharply and to remove inhibiting factors as completely as possible, the clumps were placed, at the end of the specified time, either in a neutralizing fluid or in water. In all cases, the clumps were washed through four solutions, the last two being 0.9 percent salt solutions. The cultures used were, in the main, young, generally from three to four weeks old, and in all cases human tubercle bacilli. As the clumps showed a tendency to break up in the fluids so that it was difficult and time-consuming to handle them, and contaminations frequently occurred, several methods of confining the clumps were tried, a gauze bag method being finally adopted. Draw-strings were run into a small square of gauze so that, after placing the clumps of tubercle bacilli on it, it could be drawn up into a small pouch. These gauze bags were, of course, thoroughly sterilized in Petri dishes before use. At the beginning of the experiment, clumps of culture of approximately equal size were placed in the bags, which were drawn up tightly and placed in the disinfectant solution, of which a uniform, large amount was always used. At the end of the desired time the bag was removed with sterile platinum forceps and thoroughly washed as before stated. It was then opened and the contents floated out into a small tube of sterile salt solution. The bits of culture were then seeded on slants of 2 percent glycerin agar, 0.8 percent acid to phenolphthalein. As we realized that objection might readily be raised against this method, on account of the difficulty of penetration of the disinfectant into the clumps, and especially on account of the difficulty of equal penetration of all clumps, since some of course broke up more readily than others, our results by this cruder method of culture tubes were in all cases controlled with animal cultures. For these we used the garnet method suggested by Krönig and Paul.¹⁴ Garnets of approximately equal size were soaked in a thin filtered suspension of human tubercle bacilli such that very few, if any, bacilli were present in clumps. They were then dried over CaCl_2 . About thirty infected

garnets were then placed on small platinum baskets and immersed in the various disinfectant dilutions, of which 50 c.c. were allowed for each basket. At the end of the desired times, the baskets were removed to dishes containing large quantities of distilled water, then either to ammonium sulphid or to another dish of distilled water, and then washed in two salt solutions. Ten of the treated and washed garnets were then dropped into test tubes each containing 2 c.c. of sterile salt solution and shaken in a shaking machine for five minutes. The fluid containing the organisms, which had been shaken from the garnets, was then injected subcutaneously into guinea-pigs. For each time and each dilution four tube cultures and three guinea-pigs were used, a sufficient number, it was believed, to admit of a trustworthy judgment being made from consistent results, whether positive or negative. For controls, in each experiment, clumps and garnets were treated in the same way, 0.9 percent salt solution being used in place of the disinfectant, and both tube and animal cultures being made. It may be stated here, that in not a single case out of all the series used did a control guinea-pig fail to develop local and general tuberculosis, nor a control tube culture fail to show luxuriant growth. It may, therefore, fairly be assumed that lack of growth in the four tubes and the three animals may be ascribed to a bactericidal influence of the disinfectant used. With nearly all the disinfectants the experiment was repeated two to four times with the tube cultures, and when the first animal experiment gave non-uniform results, that too was repeated. Altogether approximately one thousand guinea-pig cultures and many more tube cultures have been employed to establish our results, which are given, in brief, in Table 1. The minimum time of one hour was chosen for convenience, but with phenol a five-minute interval was also employed in the animal experiments and found to give approximately the same results. In Table 1, T. is used for tube culture; A for animal culture; percentage dilutions are indicated at the head of the columns; + indicates a growth of the culture in all four tubes; \pm or \mp indicates variation in results, the upper sign suggesting which predominates. In the animal column two signs are used, the first for local tubercle and the second for general involvement. Thus ++ means both local and general tuberculosis, while +— means local processes but no general. The \pm and \mp signs are used in the same way as was explained for the tube cultures.

The test tube cultures were kept two months or more before the final report was made, although the control tubes were well developed in one month or less. The animals were not considered as positive unless definite tubercles developed. This strict standard was necessary as an epidemic of pneumonia infection prevailed among our animals, causing pleurisy, pericarditis and peritonitis, as well as the pulmonary involvement which might simulate the more diffuse forms of tuberculosis. It was not possible with the assistance available to examine microscopically all the animals used, but tissues from doubtful cases were sectioned and examined for typical structural changes, and smears were stained and searched for tubercle bacilli. We attempted to use a sufficient number of dilutions so that at one extreme all results should be positive, while at the other they should all be negative. With some reagents that has been impossible.

Table 1 shows that phenol in 5 percent solutions is an efficient tuberculocide and nearly as efficient in 1 percent solution, though one of the guinea-pigs developed tuberculosis from bacilli which had been treated for one hour with 1 percent phenol in aqueous solution. It may be stated that guinea-pigs were also inoculated with bacilli which had been exposed only five minutes to phenol solutions with results practically the same as from a longer exposure. Formaldehyd kills human tubercle bacilli in 5 percent and 1 percent solutions, and shows considerable bactericidal power down to 0.05 percent solution. Ethyl alcohol kills tubercle bacilli in dilutions down to 25 percent, but below that it cannot be depended on. The tuberculocidal power of CuCl_2 is low, 10 percent and 5 percent in twenty-four hours killing the organisms when dried in a thin layer on the garnets; but even a practically saturated 25 percent solution fails to destroy the organisms in small clumps. HgCl_2 has a fairly high bactericidal power; 0.04 percent destroys the power of growth in the test-tube, but not the power of growth in the animal body. After twenty-four hours' exposure however, a dilution as high as 0.0001 percent destroys the power of growth even in the animal body. No higher dilutions have been tried, so that even weaker solutions might have the same effect. The HgCl_2 was on all cases neutralized with ammonium sulphid. One percent gold chlorid was strongly reduced, and deeply colored the bacterial clumps. The bacilli exposed for either one hour or twenty-four hours to 1 percent solution failed to grow either on glycerin agar or in the animal body. One-tenth percent also killed

TABLE 1

RESULTS OF EXPERIMENTS WITH DISINFECTANTS ON TUBE AND ANIMAL CULTURES

TABLE 3

COMPARISON OF THE RESULTS OF OTHERS ON THE DISINFECTANT ACTION OF THE SUBSTANCES USED IN THE EXPERIMENTS ON THE TUBERCLE BACILLUS

Organisms	Phenol		Formaldehyd		Alcohol		Chloroform		Ether		Acetone		Toluol		Lugol's		Cu Cl		Hg Cl		Au Cl		Au (CN)		Ag NO	
	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time	Per-centage	Time
Streptococcus	5	1 min.	40 0.1	1 min. 20 hr.	5 30	20 hr. 10 min.	100	1 min.	100	1 min.	10	48 hr.	1-1/400	1 min.	0.1	20 hr.	0.2	30 min.
Gonococcus	5 1	1 min. 30 min.	40 0.1	1 min. 20 hr.	10 20	30 min. 10 min.	100	1 min.	100	1 min.	0.25	15 min.	1-1/400	1 min.	0.1	20 hr.	0.2	30 min.	0.1	1 min.
M. pneumoniae	5	1 min.	40 0.1	1 min. 20 hr.	30 70	20 hr. 1 min.	100	1 min.	100	1 min.	1-1/400	1 min.	0.1	20 hr.	0.2	30 min.	0.1	1 min.
B. typhosus	.7-.8 5	15 min. 1 min.	40 0.1	1 min. 20 hr.	30	10 min.	100	1 min.	100	1 min.	40	3 hr.	0.25	5 min.	1-1/400	1 min.	0.1	20 hr.	0.2	30 min.	0.1	1 min.
B. coli	5	5 hr.	1 4	3 hr. 1 hr.	5.6	1.5† 0.6‡	4† 2.3‡	7.2†	10	4 hr.	1	3 hr.	0.1	1 min.
Staphylococcus	0.9	15 min.	0.6‡	10	17 days	3.36		27	9 hr.
B. anthracis (spores)	5	2 hr.	5	1 hr.*	25	1.69 0.42	12 min. 30 min.	3.4°	33 hr.	0.08	3 min.
B. tuberculosis	1	5 min.	0.3	1 hr.	25	1 hr.	0.5 100	24 hr.	1-2/300 §	5	24 hr.	0.1 0.001	1 hr. 24 hr.	1 0.005	1 hr. 24 hr.	0.1	24 hr.	44.25 0.01	2 hr. 24 hr.

* Seven colonies. † Solution. ‡ Vapor. § Not killed in 24 hours.

|| Did not kill in 10 days. ° (HAu Cl₄)

the organisms dried on beads in twenty-four hours, but did not have that power over clumps of bacilli, nor in a shorter time. $\text{Au}(\text{CN})_3$ in spite of its strong inhibiting power, as reported by Rosenthal, has even less bactericidal power than AuCl_3 . One hundredth percent solutions of AgNO_3 kill tubercle bacilli in twenty-four hours, but the action of this salt is relatively slow, and twenty-four hours is required to prevent the growth in the animal body of even those bacilli exposed to 1 percent solutions. The clumps, which are deeply stained by the silver, will not readily grow on artificial media.

Table 2 represents the lowest molecular concentration at which the tubercle bacilli were killed. On a molar as well as on a per-

TABLE 2
LOWEST MOLECULAR POWER AT WHICH THE TUBERCLE BACILLI WERE KILLED

Molecular Concentration	Tube Cultures		Animals	
	1 Hour	24 Hour	1 Hour	24 Hour
0.00004	Hg Cl_2
0.00006	Ag NO_3
0.00015	Hg Cl_2	Au Cl_3
0.0003	$\left\{ \begin{array}{l} \text{Au Cl}_3 \\ \text{Ag NO}_3 \end{array} \right.$
0.0007	Hg Cl_2
0.003	Ag NO_3	Formaldehyd
0.004	$\text{Au}(\text{CN})_3$
0.015	Hg Cl_2
0.02	Formaldehyd
0.03	Au Cl_3	Phenol	Au Cl_3	Phenol
0.05	Phenol	Toluol
0.08	Phenol
0.1	Formaldehyd
0.17	Formaldehyd
0.76	Cu Cl_2
4.04	Alcohol	Alcohol	Alcohol	Alcohol

centage basis, as may be seen from the table, HgCl_2 holds the highest place as a tuberculocide. This salt has the highest disinfectant power of any of the metallic salts used by us. Koch,¹⁵ in his work on disinfection in 1881, also assigns the highest disinfectant value to HgCl_2 . Post and Nicoll¹⁶ assign it a similar place in their experiments with rapidly growing organisms, but they do not neutralize nor even wash out their disinfectant and so inhibition must be added to the bactericidal effect. Krönig and Paul, however, using the garnet method and neutralizing the disinfectant, and then thoroughly washing the garnets, found that 0.42 percent HgCl_2 killed anthrax spores

15. Reviewed in *Mitt. a. d. Kaisl. Gsndhtsamt.*, 1881, p. 234.

16. *Jour. Am. Med. Assn.*, 1910, 55, p. 1635.

in thirty minutes, while it required two hours for 4.25 percent AgNO_3 to kill them, and 3.36 percent CuSO_4 did not kill them all even after ten days' exposure. Neither of these workers report results with the gold salts, and so no comparison can be made. Krönig and Paul, however, state that gold cyanid has very little disinfectant power and that gold chlorid has more influence. This statement corresponds with our findings regarding the tuberculocidal action of these gold salts.

Table 3 gives in brief the results which we have been able to find in the literature on the disinfectant action of the substances used by us, compared with our own results on the tubercle bacillus. In these reports of other workers, the methods vary greatly, and inhibitory and disinfectant influence are not always sharply distinguished. Leaving these points out of consideration, the bacillus tuberculosis appears less resistant to phenol, formaldehyd, HgCl_2 , AgNO_3 , and to AuCl_3 than the other organisms in the table, if we may compare its action to that which Krönig and Paul give for HAuCl_4 , which is probably a dissociation product of AuCl_3 . On the other hand, the tubercle bacillus is more resistant to alcohol, chloroform, ether, acetone, toluol, and the iodine-potassium-iodid mixture known as Lugol's solution. Omitting phenol of the first group and the iodine solution of the second, we may say that the human tubercle bacilli are less resistant to the disinfectants which are not fat-soluble, and more resistant to those which are especially fat soluble. The phenol exception may be explained by Cooper's statement¹⁷ that the disinfectant action of phenol is due to its power to precipitate the proteins of the bacterial cell.

It has long been known that tubercle bacilli were especially rich in fats. Wells¹⁸ states that different workers find an amount of substance soluble in fat solvents varying from 20 to 40 percent of the weight of the bacilli. Kresling¹⁹ found the following percentages of chloroform soluble material:

Free fatty acid.....	14.38
Neutral fats and fatty acid esters.....	77.25
Alcohols from fatty acid esters.....	39.10
Lecithin	0.16

Deycke²⁰ states that these bacteria have not only a fatty sheath but also fat penetrating the body. He regards acid fastness as due to free fatty acids and finds that tubercle bacilli freed from their fats are no longer acid fast.

On the basis of their large fat content, it has naturally been assumed that fat solvents would more readily penetrate these organisms. It was shown by Sher-

17. *Jour. Biochem.*, 1909, 7, p. 175.

18. *Chemical Pathology*, 1914, Chap. 2.

19. *Centralbl. f. Bakteriöl.*, Abt. 1, Orig., 1901, 30, p. 897.

20. *München. med. Wchnschr.*, 1910, 57, p. 633.

man²¹ that fat soluble dyes, while readily staining masses of tubercle bacilli, almost entirely failed to stain individual organisms, and it was shown by De Witt²² that many non-fat-soluble dyes, as the methylene blue group, penetrated and stained the individual tubercle bacilli very well. Benians²³ stated that tubercle bacilli were killed if shaken for one hour with 10 percent of toluene, while the bacillus coli required four hours, and the staphylococcus aureus seventeen days, with 10 percent toluene. Staphylococci were killed after being exposed for twelve days to benzol and five weeks to xylene, and the bacillus coli was killed after being shaken with benzol one hour, and five hours with xylene. Sata²⁴ and Ritchie²⁵ were unable to demonstrate fats in the staphylococcus aureus by the use of fat dyes, except when grown on glycerin agar, and Sata found no fat in the bacillus coli when grown on any media. Wells and Corper⁸ also found that toluene water killed tubercle bacilli. These results but strengthened the idea which was expressed in Benians' paper that "bacteria having an available lipoid moiety are readily destroyed by toluene, and those not having such a moiety escape its action." On the other hand, Rosenau²⁶ states that it is doubtful whether the fatty substances of the tubercle bacillus protect it against external influences, and Bürgi²⁷ says that the connection of lipoid solubility with disinfectant power has not been proved.

Our own results with toluene have been negative with the exception of the 0.5 percent, which in twenty-four hours was able to destroy the organisms on the garnets so that they failed to infect guinea-pigs. As toluene is but slightly soluble in water this was a super-saturated solution, or rather an emulsion. The pure toluene in the same time failed to kill the tubercle bacilli even in a thin layer on the garnets. These results with the toluene emulsion in water agree, then, fairly well with those of Benians, and of Wells and Corper. It is difficult to understand why the pure toluene should not have had a similar effect, unless, as has been suggested, the pure toluene may have precipitated a layer on the surface and so it was unable to penetrate to the deeper layers. The tube cultures of the bacteria, exposed to full strength toluene showed but little development, and so considerable tuberculocidal action must be assigned to toluene. Chloroform, ether, and acetone, however, had no such contradictory action. The tube cultures of the organisms exposed to full strength acetone and chloroform showed only slight growth, but all animals developed local and general tuberculosis. Therefore the disinfectant action of these fat solvents must be considered as lower on tubercle bacilli than on fat-poor organisms, and much lower on tubercle bacilli

21. *Jour. Infect. Dis.*, 1913, 12, p. 249.

22. *Jour. Infect. Dis.*, 1913, 12, p. 68.

23. *Ztschr. f. Chemoth.*, 1913, Orig., 2, p. 40.

24. *Centralbl. f. allg. Pathol.*, 1900, 11, p. 97.

25. *Jour. Path. and Bacteriol.*, 1904, 10, p. 334.

26. *Bull. Hyg. Lab., U. S. P. H. and M. H. S.*, 1909, No. 57.

27. *Handb. d. path., Mikroorganismen*, 1913, p. 534.

than that of many reagents which are insoluble in fats. For this reason such disinfectant action as toluene has on tubercle bacilli must be ascribed to some other cause than solubility.

Considerable question has been raised in regard to the concentration of alcohol which has bactericidal influence, Beyer²⁸ stating that dilutions from 60 to 80 percent by weight have the greatest disinfectant value, and that below 60 percent and above 80 percent there is practically no disinfection. He found that silk threads infected with the staphylococcus aureus and exposed for six days to absolute alcohol, protected from evaporation, were uninjured. Schaumburg²⁹ suggested, as an explanation of the unfavorable results previously obtained with absolute alcohol, that alcohol precipitates the proteins on the surface and thus prevents penetration to the deeper layers. He finds that if the organisms are exposed in the form of an emulsion they are quickly killed by absolute alcohol, although, considering the form in which the organisms are exposed, the "absolute"-ness of the alcohol might well be questioned after mixture with the emulsion.

Krönig and Paul¹⁴ concluded from a long and exhaustive series of experiments, that disinfectant action of many substances, especially acids, alkalies and metallic salts, depended on dissociation. This statement has been accepted by most of the disinfection workers since that time. Burgi²⁷ states that dissociation forms the most important preliminary in the disinfectant action of electrolytes. In easily dissociating mercury preparations, he says, the mercury ions disinfect most, the anions have the second place in influence, and the non-dissociated fraction of the molecule stands lowest in disinfecting power. There are, however, complicated organic mercury preparations in which the whole molecule has strong bactericidal influence. Reichel³⁰ refers the disinfectant action of HgCl_2 to the "Hg ion, whose strong affinity for proteins must lead to an irreversible reaction." Unfortunately, in the case of most of the metallic salts used by us, no reliable data are at hand regarding their degree of dissociation. Doubt has been cast on the accuracy of some of the earlier work, especially as regards the mercury salts. Chick gives the Hg ion concentrations, as obtained from the tables of Luther and Kahlenberg, as follows:

Percentage	Hg ions
0.1.....	63.0
0.05.....	57.5
0.01.....	42.5
0.005.....	37.0
0.001.....	23.0
0.0005.....	16.5

It is generally recognized that the degree of dissociation of HgCl_2 is by no means proportional to its concentration, and Chick and Martin³¹ state that 100 percent increase in percentage concentration of the salt increases the Hg ion concentration only about 15 per cent. Later work has also shown that the electrolytic dissociation is not at all so simple as was earlier thought, and that many of the metallic salts, notably those of gold and of mercury, form many complex ions instead of, or as well as, the simple metal ion and the anion.

28. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1911, 70, p. 225.

29. *Deutsch. med. Wchnschr.*, 1912, 38, p. 403.

30. *Biochem. Ztschr.*, 1909, 22, p. 149.

31. *Jour. Hyg.*, 1908, 8, p. 654.

Krönig and Paul as early as 1897 recognized that the gold salts dissociated but little, an observation which has recently been verified to the extent that probably but few simple gold ions are formed in the process of electrolytic dissociation. Data are given, which are as yet accepted, on the degree of dissociation of AgNO_3 and to some extent of CuCl_2 .

	Percentage of Concentration	Molecular Concentration	Degree of Dis-sociation	Concentration of Metallic Ions
AgNO_3	1	.0058	95.28	.0056
	0.5	.0029	96.81	.0028
	0.1	.000588	98.72	.00057
	0.075	.000441	98.90	.00044
	0.05	.000294	99.10	.00029
	0.025	.000147	99.41	.00015
	0.01	.000059	99.71	.000059
CuCl_2	25.99	1.524	34.1	.5197
	12.995	.762	50.1	.3818
	2.6	.152	71.2	.1082
	1.3	.076	77.6	.0045
	.26	.015	90.1	.0135

These figures are too few to make comparisons safe and no statement could be made, based on our work as to whether the metallic ion is the true disinfecting factor or not. We may say only that many of the metallic salts, notably those of mercury and silver, are reliable tuberculocides, although they are slower than phenol, formaldehyd, and alcohol in their action.

SUMMARY AND CONCLUSIONS

Phenol in 5 per cent water solution kills human tubercle bacilli in five minutes, one hour, six hours, and twenty-four hours. It is nearly as efficient in 1 per cent solution, and shows some tuberculocidal action down to 0.1 per cent solution.

Formaldehyd in 1 per cent solution kills all tubercle bacilli in one hour (shorter time not tested). In 0.01 per cent solution it kills in twenty-four hours and so no disease develops in guinea-pigs. Formaldehyd, therefore, is somewhat more efficient than phenol.

Ethyl alcohol in 25 per cent solution kills all tubercle bacilli within one hour (shorter time not tried).

Acetone, chloroform and ether have very little, if any, tuberculocidal influence. Toluene and iodine show slight influence.

Of the metallic salts used, mercuric chlorid shows the greatest tuberculocidal action, 0.001 per cent killing in twenty-four hours, and 0.1 per cent. in one hour. Gold chlorid in 0.005 per cent solution

kills in twenty-four hours, while 0.025 per cent silver nitrate kills in the same time. One-tenth per cent gold tri-cyanid and 5 per cent copper chlorid kill the organisms in twenty-four hours.

From a comparison of the results of the experiments contained in this paper with those of disinfection work on other more rapidly growing organisms, the bacillus tuberculosis appears less resistant than the streptococcus, staphylococcus, pneumococcus or gonococcus, or than the bacillus typhosus, coli or anthracis spores, to phenol, formaldehyd, HgCl_2 , AgNO_3 , and AuCl_3 , but more resistant than these other organisms to alcohol, chloroform, ether, acetone, toluene, and Lugol's solution.

The fat content of the tubercle bacillus does not determine its resistance to disinfectants. Our experiments seem to show that if the comparatively high content of this organism differentiates its behavior from that of bacteria of low fat content, it does so by rendering the tubercle bacillus more resistant to fat solvents, and less resistant to substances insoluble in fats.

THE STANDARDIZATION OF ANTIHOG-CHOLERA SERUM *

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The use of antihog-cholera serum is of great importance to the live-stock industries. Since its discovery by Dorset, McBride and Niles in 1908, its use has constantly increased. The potency of the antihog-cholera serum has been tested in the United States by administering serum to susceptible pigs and inoculating them with virulent blood, at the same time giving two or more pigs the same dose of virulent blood alone. If the pigs receiving both serum and virulent blood survive, and those receiving the virus only readily succumb, the serum is declared potent and fit for use in the simultaneous method in doses slightly larger than those used in the test.

Our object has been to ascertain if the various sources of error, affecting the methods of standardization of antihog-cholera serum, may be reduced or eliminated.

The first experiment was carried on to discover whether the hog-cholera serum retained its potency when dried. A quantity of serum was dried in a Faust dryer at a temperature below 40 C. After about two weeks a portion was redissolved in sterile water in such a manner that it was approximately restored to its original volume, and it was then administered intramuscularly to 10 pigs. Each pig in the series was given 2 c.c. of fixed virus of a strain obtained from Dr. John Reichel. The doses of serum were between the limits of 0.05 to 0.4 c.c. per pound, as suggested by Fitzgerald and Fischer. The results of the test are summarized in Table 1.

It is seen that up to the dose of 0.3 c.c. per pound a part of the pigs sickened, and that at 0.4 c.c. all of the pigs were protected. Throughout the remainder of this paper the dose of serum which protects all of the pigs from visible symptoms of disease and from continued temperatures above 104.4 F. will be called the P + dose, the dose which just fails to protect will be termed the P — dose. In these experiments

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TABLE 1
RESULTS OF EXPERIMENTS OF KC CLEAR SERUM AND VIRUS (FIG 7)

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
34	0.5 c.c.	7	107.2	Died in 11 days
35	0.5 c.c.	6	107.3	Died in 9 days
		Average 6.5		
36	0.1 c.c.	8	106.0	Recovered
37	0.1 c.c.	6	106.1	Died in 7 days
		Average 7		
38	0.2 c.c.	15	106.1	Recovered, symptoms severe
39	0.2 c.c.	2	105.7	Recovered, slight symptoms
		Average 8.5		
40	0.3 c.c.	11	106.4	Killed, chronic
41	0.3 c.c.	0	104.2	Recovered, no symptoms
		Average 5.5		
42	0.4 c.c.	0	104.4	Recovered, no symptoms
..	0.4 c.c.	1	104.7	Recovered, no symptoms
		Average 0.5		

The dose of virus was 2 c.c. in each case.

hogs with temperatures of below 104.5 F. have not usually shown many symptoms, except some cases which tended to become chronic. Therefore, 104.5 F. was arbitrarily chosen as the line of demarcation

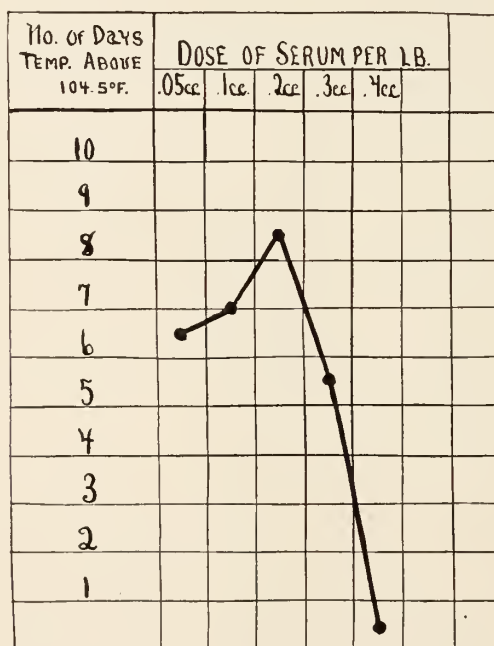


Fig. 1.—The effect of the quantity of serum on the length of time the temperature remained above 104.5 F.

between a slight and definite febrile reaction following the vaccination. The exact temperature chosen does not affect the magnitude of the P + and the P — doses. Considerable attention will be given in the future to establishing the best temperature. In these tests, 104.5 F., however, will be used throughout. Since the data are comparative, the exact point chosen theoretically as well as practically does not affect the accuracy of the results. The pigs receiving doses of serum of the same size formed a group. The number of days' duration of elevated temperature for each group was averaged. When these averages are considered in place of the individual records, considerable regularity is seen in the results. The significance is seen when the doses of serum are plotted on the horizontal axis and the number of days on the vertical (Chart 1).

EFFECT OF DESICCATION AND CENTRIFUGALIZATION

The next test carried out was undertaken to determine whether the serum lost strength in desiccation; also to ascertain if the removal of the red blood-cells affected the magnitude of the P + dose.

Twenty-four hogs were bled from the tail into a covered receptacle. After defibrination 25 per cent. of the blood was preserved with 1 per cent. chloro-

TABLE 2
RESULTS OF EXPERIMENTS WITH WHOLE SERUM AND FIXED VIRUS (PIG 65)

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
82	0.05 c.c.	5	105.8	Recovered
97	0.05 c.c.	5	104.8	Recovered, slight symptoms
117	0.05 c.c.	5	108.0	Died in 12 days
139	0.05 c.c.	10	107.0	Died in 22 days
		Average 6.2		
83	0.1 c.c.	7	105.2	Recovered
98	0.1 c.c.	5	105.2	Recovered
110	0.1 c.c.	11	106.2	Recovered
124	0.1 c.c.	12	107.0	Recovered
		Average 8.7		
74	0.2 c.c.	7	104.7	Recovered
87	0.2 c.c.	9	107.6	Died in 13 days
112	0.2 c.c.	13	107.0	Recovered
113	0.2 c.c.	3	105.2	Recovered
		Average 8, —P—		
73	0.3 c.c.	3	104.8	Recovered
84	0.3 c.c.	5	105.4	Recovered
120	0.3 c.c.	1	104.7	Recovered
129	0.3 c.c.	3	105.2	Recovered
		Average 3, —P+		
75	0.4 c.c.	1	104.6	Recovered
119	0.4 c.c.	0	103.2	Recovered
103	0.4 c.c.	6	105.3	Recovered
134	0.4 c.c.	4	104.3	Recovered
		Average 2.75		

The dose of virus was 2 c.c. in each case.

TABLE 3

RESULTS OF EXPERIMENTS WITH CENTRIFUGATED SERUM AND FIXED VIRUS (FIG 65)

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
66	0.05 c.c.	5	106.1	Recovered
155	0.05 c.c.	13	107.7	Recovered
111	0.05 c.c.	5	106.2	Died in 21 days
138	0.05 c.c.	8	107.0	Died in 31 days
		Average 7.7		
86	0.1 c.c.	8	106.0	Died in 29 days
99	0.1 c.c.	5	104.7	Recovered
105	0.1 c.c.	9	106.9	Recovered
123	0.1 c.c.	13	107.5	Died in 21 days
		Average 8.7,—P—		
71	0.2 c.c.	3	105.1	Recovered
132	0.2 c.c.	2	104.8	Recovered
93	0.2 c.c.	1	105.0	Recovered
106	0.2 c.c.	2	104.7	Recovered
		Average 2,—P+		
80	0.3 c.c.	1	104.8	Recovered
121	0.3 c.c.	5	105.2	Recovered
118	0.3 c.c.	1	104.8	Recovered
126	0.3 c.c.	0	104.4	Recovered
		Average 1.7		
68	0.4 c.c.	3	105.1	Recovered
76	0.4 c.c.	2	104.8	Recovered
109	0.4 c.c.	0	104.0	Recovered
133	0.4 c.c.	0	104.2	Recovered
		Average 1.2		

The dose of virus was 2 c.c. in each case.

TABLE 4

RESULTS OF EXPERIMENTS WITH DESICCATED SERUM AND FIXED VIRUS (FIG 65)

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
67	0.05 c.c.	13	107.8	Died in 16 days
91	0.05 c.c.	12	105.9	Recovered
101	0.05 c.c.	10	105.8	Recovered
137	0.05 c.c.	14	107.7	Died in 21 days
		Average 12		
102	0.1 c.c.	9	106.7	Recovered
122	0.1 c.c.	7	106.4	Recovered
81	0.1 c.c.	3	105.4	Recovered
96	0.1 c.c.	6	105.9	Recovered
		Average 6.2,—P—		
85	0.2 c.c.	0	104.1	Recovered
92	0.2 c.c.	1	104.6	Recovered
100	0.2 c.c.	1	104.5	Recovered
128	0.2 c.c.	5	105.6	Recovered
		Average 1.7,—P+		
72	0.3 c.c.	5	104.8	Recovered
135	0.3 c.c.	3	105.1	Recovered
116	0.3 c.c.	4	106.3	Recovered
125	0.3 c.c.	2	104.8	Recovered
		Average 3.3		
77	0.4 c.c.	0	104.0	Recovered
70	0.4 c.c.	1	104.6	Recovered
115	0.4 c.c.	2	105.0	Recovered
..	0.4 c.c.	3	105.2	Recovered
		Average 1.5		

In each case the dose of virus was 2 c.c.

TABLE 5
RESULTS OF EXPERIMENTS WITH WASHED CORPUSCLES AND FIXED VIRUS (FIG 65)

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
88	30 c.c.	9	107.0	Died in 12 days
136	30 c.c.	9	107.0	Killed moribund in 10 days
94	50 c.c.	8	106.7	Died in 10 days
75	75 c.c.	4	106.8	Recovered

In each case the dose of virus was 2 c.c.

form and saved without further treatment. The remaining 75 per cent. of the blood was freed from corpuscles by centrifugalization. After mixing the various lots of centrifugings together in a large enameled kettle about 1 liter was bottled and the remainder desiccated. In addition to this a certain percentage of the total quantity of red blood-cells from each bleeding was twice washed with sterile salt solution. Defibrinated blood (whole serum), corpuscle-free serum, desiccated corpuscle-free serum, and washed red blood-cells were obtained separately by these procedures. They were all from the same source and, hence, suitable for comparison. Seventy-four pigs were used in the test. Complete series, with four pigs for doses of each size, were carried out with the whole serum, the centrifuged serum, and the desiccated, centrifuged serum. Four pigs received the washed corpuscles. All pigs treated received 2 c.c. of fixed virus and eight check pigs received the virus alone. The results of these tests are summarized in Tables 2, 3, 4, 5 and 6.

These tables show that the desiccated and the centrifuged sera were approximately the same strength, as their P + and P — doses had the same magnitude. The whole serum was distinctly weaker, inasmuch as its P + dose was 0.3 c.c. per pound. The corpuscles have been found to be 40 per cent. of the entire volume of the whole serum, hence its strength should be 60 percent of the strength of centrifuged serum, assuming, as is shown in Table 5, that the washed corpuscles are practically inert. Since the ratio of the P + doses of the two sera is 2:3 the strength of the whole serum would be 66.66 + per cent. that of the centrifuged serum. The theoretical strength of the whole serum is 60 per cent. that of the centrifuged, hence the results are in close agreement. It will be seen from Table 2 that the P + dose of the whole serum is 0.3 c.c. per pound, because between the doses of 0.2 c.c. and 0.3 c.c. the average duration of temperature drops from 8 to 3 days. The highest temperature of any of the pigs on the 0.3 c.c. dose was only 105.4, showing that they were protected from serious sickness.

In Table 3, which deals with the centrifuged serum, the P + dose is 0.2 c.c. per pound, and the P — dose 0.1 c.c. per pound, because here the average drops from 8.7 to 2 days.

In Table 4 the P + dose of the desiccated serum is 0.2 c.c. per pound as evidenced by the average which dropped from 6.2 to 1.7 days. The rise to 3.3 days on the dose of 0.3 c.c. per pound is not significant, since a few days temperature above 104.5 is frequently observed in all doses investigated.

It is noted that 30 c.c. of washed corpuscles, which contained two-thirds of its volume, or 20 c.c. of solid red cells, gave little protection. The 50 c.c. dose gave no protection. In the one instance where the 75 c.c. dose was used, there was apparently a little protection. This may have been the result of the trace of serum left. The corpuscles used in this experiment were washed twice with physiologic salt solution. Beginning with 100 c.c. of the defibrinated blood, which by the hematocrite showed 40 per cent. of corpuscles, it was found that 50 c.c., or five-sixths of the amount of serum, could be removed at the first centrifuging. This left 10 c.c. of serum and 40 c.c. of corpuscles. The original volume was restored with the salt solution, and the corpuscles thrown down again. Five-sixths of the total amount of liquid was removed and hence five-sixths of the remaining 10 c.c. of serum, leaving 40 c.c. of corpuscles and 1.66 c.c. of serum. Repeating the process, five-sixths of the 1.66 c.c. of serum was removed, leaving 0.27 c.c. of serum in the 50 c.c. residue after removing the last wash-water. These figures are, probably, a little low, as there is difficulty in removing all the serum adhering to the red cells. Reference to the tests of serum freed from the corpuscles shows that pure serum is considerably stronger.

TABLE 6
RESULTS OF EXPERIMENTS WITH THE VIRUS (PIG 65)

No. of Pig	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
79	8	106.6	Killed moribund in 12 days
89	8	107.2	Killed moribund in 9 days
95	9	107.5	Killed moribund in 10 days
108	9	108.2	Killed moribund in 11 days
107	13	107.6	Killed moribund in 17 days
154	8	107.8	Killed after 8 days, severely affected
130	7	107.5	Killed after 8 days, severely affected
114	6	108.1	Killed after 9 days, severely affected
Average 8.5			

In each case the dose of virus was 2 c.c.

The eight pigs used in this experiment all rapidly developed acute hog-cholera. These pigs were selected from the four lots of pigs used in the preceding experiment. Two pigs came from each lot. This demonstrates that the pigs used were susceptible and the virus active.

THE EFFECT OF ALUMINUM ON SERUM

The third series of tests was carried out to ascertain if aluminum is a suitable metal in which to store serum. As antihog-cholera serum is produced in very large amounts there is need of some container in which large quantities may be mixed together. Covered vessels up to 250 gallons capacity may be constructed out of seamless aluminum and so arranged that they may be sterilized by heat. As aluminum is non-toxic and non-corrosive and readily cleaned it would seem to be admirably adapted for this use.

In this test the aluminum was in contact with the serum for twenty-four hours with constant shaking. This is several times longer than the serum would remain in contact with the aluminum in the mixing process.

A quantity of desiccated anti-hog-cholera serum was dissolved and one-half placed in an aluminum flask, and the other half put into a glass flask. Both were shaken for twenty-four hours at room temperature. A quantity of

TABLE 7
RESULTS OF EXPERIMENTS OF STANDARD AND FILTERED VIRUS

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
292	0.02 c.c.	2	105.4	Recovered
291	0.02 c.c.	3	105.2	Recovered
332	0.02 c.c.	4	106.0	Recovered
		Average 3		
320	0.05 c.c.	7	107.2	Killed moribund in 10 days
304	0.05 c.c.	0	103.8	Recovered
305	0.05 c.c.	0	103.6	Recovered
		Average 2.33		
315	0.1 c.c.	3	105.8	Recovered
312	0.1 c.c.	10	107.0	Killed moribund in 11 days
318	0.1 c.c.	6	107.0	Still sick at end of 3 weeks
		Average 6.33		
336	0.2 c.c.	2	105.2	Recovered
339	0.2 c.c.	1	104.8	Recovered
342	0.2 c.c.	0	103.6	Recovered
		Average 1 P+		
345	0.3 c.c.	3	104.9	Recovered
348	0.3 c.c.	1	104.9	Recovered
351	0.3 c.c.	3	106.2	Recovered
		Average 2.33		
354	0.4 c.c.	0	103.6	Recovered
357	0.4 c.c.	0	104.4	Recovered
360	0.4 c.c.	0	104.2	Recovered
		Average 0		

In each case the dose of filtered virus was 15 c.c.

TABLE 8

RESULTS OF EXPERIMENT WITH ALUMINUM TREATED SERUM AND FILTERED VIRUS

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
301	0.02 c.c.	9	107.0	Killed moribund in 11 days
324	0.02 c.c.	8	107.6	Killed moribund in 10 days
296	0.02 c.c.	0	103.2	No symptoms
		Average 5.66		
298	0.05 c.c.	7	105.6	Recovered
314	0.05 c.c.	1	105.6	Recovered
319	0.05 c.c.	10	107.2	Died in 12 days
		Average 6		
306	0.1 c.c.	9	107.4	Died in 21 days
303	0.1 c.c.	0	104.4	Recovered
325	0.1 c.c.	0	107.0	Died in 11 days
		Average 6 P —		
337	0.2 c.c.	3	104.6	Recovered
340	0.2 c.c.	0	104.4	Recovered
343	0.2 c.c.	1	105.0	Recovered
		Average 1.33 P+		
346	0.3 c.c.	1	104.7	Recovered
349	0.3 c.c.	0	103.7	Recovered
352	0.3 c.c.	0	104.3	Recovered
		Average 0.33		
355	0.4 c.c.	1	105.6	Recovered
358	0.4 c.c.	0	104.0	Recovered
361	0.4 c.c.	2	105.0	Recovered
		Average 1		

In each case the dose of filtered virus was 15 c.c.

TABLE 9

RESULTS OF EXPERIMENTS WITH K. C. SERUM AND FILTERED VIRUS

No. of Pig	Dose of Serum per Pound	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
308	0.02 c.c.	0	104.3	Recovered
328	0.02 c.c.	6	106.0	Died in 9 days
333	0.02 c.c.	10	107.0	Killed moribund in 10 days
		Average 5.3		
313	0.06 c.c.	10	107.0	Killed moribund in 13 days
329	0.06 c.c.	7	107.0	Killed moribund in 10 days
317	0.06 c.c.	7	108.0	Killed moribund in 9 days
		Average 8		
293	0.01 c.c.	9	105.6	Recovered
322	0.01 c.c.	6	107.0	Died in 11 days
310	0.01 c.c.	5	107.9	Still somewhat sick at end of 21 days
		Average 6.66		
338	0.2 c.c.	4	105.2	Recovered
341	0.2 c.c.	5	105.8	Recovered
344	0.2 c.c.	9	107.4	Died in 11 days
		Average 6		
347	0.3 c.c.	1	105.6	Recovered
350	0.3 c.c.	6	106.2	Recovered
353	0.3 c.c.	0	104.0	Recovered
		Average 2.33		
356	0.4 c.c.	2	105.0	Recovered
359	0.4 c.c.	0	104.0	Recovered
363	0.4 c.c.	4	105.5	Recovered
		Average 2		

serum from another source was dried in a Faust dryer. The three sera were injected into three series of test pigs on the same day against the same virus. Weights were balanced, but owing to an error it was impossible to balance origin of pigs. The results are set forth in Tables 7, 8, 9 and 10.

TABLE 10
RESULTS OF EXPERIMENTS WITH FILTERED VIRUS

No. of Pig	No. of Days Temperature Above 104.4 F.	Highest Temperature	Results
288	10	106.0	Died in 14 days
316	9	107.2	Killed moribund in 10 days
311	7	107.6	Died in 8 days
	Average 8.66		

In each case in both Tables 9 and 10 the dose of filtered virus was 15 c.c.

Tables 7 and 8 show that the P + dose of the serum is the same, 0.2 c.c. per pound, when the serum is kept in glass and when the same serum is treated with aluminum.

Therefore we may conclude that, within the limits of accuracy of the method, the aluminum showed no deleterious action on the serum.

Table 9 shows that the K. C. serum has a P + dose of 0.3 c.c. per pound, and therefore must be used in 50 percent greater doses than the standard serum.

Table 10 shows that the filtered virus used was quite virulent.

PRINCIPLES OF STANDARDIZATION

These experiments indicate that, for scientific purposes at least, considerable accuracy may be attained in the standardization of anti-hog-cholera serum.

If it were not for the varying strength of the hog-cholera virus and the various grades of susceptibility exhibited toward hog-cholera by pigs of different breeds, ages, weights and sources, the determination of the P + dose might be sufficient standardization for a serum. However, in order to accurately standardize a serum, these variables must be eliminated. In addition to the irregular susceptibility of hogs to natural and artificial infection with hog-cholera, the filterable virus as shown by Uhlenhut varies in strength quite rapidly when stored, and the antihog-cholera serum, in the liquid form, probably gradually loses its strength through the action of light, heat, oxygen and moisture. The effect of these disturbing factors causes a variation in the magnitude of the P + dose of the same serum when the determination

is made on pigs from different sources, or on pigs from the same source with different virus. The exact amount of this variation has not, as yet, been determined. The use of a standard desiccated anti-serum gives promise of eliminating these variables.

Ehrlich has shown that antisera, when completely desiccated and stored in high vacuo in a dark, cool place, remain practically unchanged in potency for long periods of time, and that samples of diphtheria antitoxin so preserved, when used as an arbitrary unit, furnish the most reliable method for the standardization of diphtheria antitoxin.

By desiccating antihog-cholera serum in an analogous manner there is every reason to believe that it becomes sufficiently stable to furnish a standard or unit by which the strength of any sample of hog-cholera serum may be more accurately measured, if the test is so arranged that one-half of the pigs are given the standard serum in graduated doses, and the other half receive the serum to be tested in doses of the same size, and all pigs receive the same virus in doses of the same size.

Three or four pigs are used on each sized dose of serum in order that the average may be as accurate as possible. The pigs are obtained from herds in which cholera has not existed, and in which vaccination has not been practiced. They are held in quarantine from ten days to two weeks in pens widely separated from infection. Their origin is noted because the susceptibility of pigs from different origins — farms — varies greatly. On the day of the test they are weighed and divided into squads in such a manner that for every pig of a certain origin used in testing the unknown serum a pig of about the same weight and the same origin, will receive the standard serum in doses of the same size. This principle of balancing weights and origins of pigs is also applied to the selection of pigs for each rate of serum, so that any difference in susceptibility of the pig will balance throughout the test and, hence, this factor will be eliminated, since the same virus has been used on all pigs. In each series the variation in the strength of the virus is likewise eliminated.

When the magnitude of the $P +$ dose of standard serum and the $P +$ dose of the serum to be tested are simultaneously determined as outlined above, the ratio of the $P +$ unknown to the $P +$ standard is independent of the virus used, and largely independent of the susceptibility of the pigs used. More accuracy is attained if large numbers of hogs are used on doses of each size and large numbers of doses tested

between the limits of 0.05 and 0.4 c.c. per pound. The limits of accuracy of the method have not been exactly determined.

Although the standardization of anti-infectious serum can probably never be so simple or so accurate as the standardization of an antitoxic serum against its antitoxin, nevertheless antitoxic sera; e. g., blackleg and swine plague sera (Schweineseuche), are standardized with considerable accuracy. The fact that we are dealing with an ultravisible virus and that we cannot determine its concentration does not seem to unusually complicate the method, as even in those diseases in which we are dealing with a visible organism, which may be raised *in vitro*, the variations in virulency are so great that the determination of the number of organisms injected is not a definite measure of the virulence of the test dose.

A future paper will deal more minutely with the various features of the proposed method of standardization.

CONCLUSIONS

When a series of pigs were inoculated with increasing serum and virus, a point was reached at which the pigs no longer showed more than a transient fever. This dose is the protective dose of serum.

The same serum tested in both the desiccated and non-desiccated forms showed the same strength.

A serum, from which the red blood-cells had been removed, was definitely more potent than the same serum containing red blood-cells.

The potency of the serum was not measurably affected by storage in aluminum for twenty-four hours.

A BIOMETRICAL STUDY OF THE MUCOSUS CAPSULATUS GROUP*

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For some time work has been carried on in an endeavor to decide if the micro-organisms now classed under the general head of mucosus capsulatus could be divided into distinct species after a biometrical review of their various characters.

So much work has been done in this group and the literature so thoroughly reviewed that only the more complete of the earlier publications need be mentioned. Fricke,¹ in 1896, gave the general characters of the group as follows: short, non-spore bearing, encapsulated bacilli, non-motile and gram-negative, showing a slimy growth on different media, not liquefying gelatin, sometimes forming indol, and fermenting certain of the carbohydrates. Clairmont,² in 1902, continued the study of the members of this group and in his work included reference to the reaction of agglutination, which however he found unsatisfactory as a means of differentiation. Perkins,³ in 1904, proposed a simple classification based on the fermentation characteristics of these micro-organisms. The first species was designated as the bacterium *aërogenes* (including all strains of the bacilli *lactis aërogenes*, *capsulatus septicus*, Pfeiffer and Howard, and a large series isolated by Perkins. This group fermented the monosaccharids—dextrose and levulose; the disaccharids—saccharose, lactose and maltose, the pentose arabinose, the triatomic alcohol glycerin, and the hexatomic alcohol mannite. The second species, the bacterium *pneumonicum*, included all the Friedländer group, the bacillus *ozenae*, probably the bacillus *rhinoscleromatis*, and fermented all carbohydrates except lactose. The third species had as its prototype the bacillus *acidi lactici*, and members of this species fermented all the carbohydrates enumerated, with the exception of saccharose.

There has been no recent attempt made to classify this group on the basis of cultural features or biochemical activities alone. Behan,⁴ however, has quite recently endeavored to differentiate these various species by means of the reaction of agglutination, with apparently some success.

Our study was undertaken in the hope that the biometrical methods employed by Winslow in the study of the coccaceae, might serve to finally elucidate the problem as to whether or not micro-organisms represent one or more species which were heretofore described as belonging to many species.

* Received for publication May 16, 1914.

1. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1896, 23, p. 380.

2. *Ibid.*, 1902, 39, p. 1.

3. *Jour. Infect. Dis.*, 1904, 1, p. 241.

4. *Centralbl. f. Bakteriöl.*, Abt. 1, Orig., 1912, 66, p. 110.

The earliest description of many of the bacteria investigated by us are utterly inadequate for the differentiation of species, for examples, Fricke's⁵ endeavor to separate the species on the basis of differences in the appearance on potato, and Clairmont's⁶ on the basis of the time of coagulation of milk. The later work of Perkins⁷ and of Strong⁸ showed the weaknesses of such criteria. Perkins, as a result of his study of this group, investigated thirty-seven strains.

This classification of Perkins assumes constant results from a study of the fermentation reactions of these species. Beham's⁹ later work is an endeavor to show that the immunity reactions, the agglutination reaction in particular, is probably of greater value in the differentiation than any cultural features or biochemical reactions. It seemed that the most satisfactory results would be obtained if a very considerable number of micro-organisms, from widely different sources, were studied in a fashion that would show: (1) the common characteristics of all the members of the group, and (2) an analysis of differences on which it might be possible to group these bacteria in various species.

In all, forty-four cultures have been studied. These have been obtained from the Král collection, from the Pasteur Institute, Paris, from the Lister Institute, London, The American Museum of Natural History, Harvard Medical School, University of Chicago, Leland Stanford Junior University, Massachusetts General Hospital, University of California, The Cutter Laboratory, University of California Hospital, and the Infirmary, University of California. Fourteen of the forty-four cultures were designated as the bacterium *capsulatus mucosus*, Fasching; five cultures as the bacterium *capsulatus Pfeiffer*; six cultures as the bacillus *aërogenes Escherich*; four cultures as the bacterium *ozenae Abel-Löwenberg*; seven as the bacillus *pneumoniae Friedländer*, five as the bacterium *rhinoscleromatis v. Frisch*, and one as the bacterium *crassus sputigenus Kreiböhm*. To further round out the group two strains of the bacterium *enteritidis Gaertner*, were also included.

Obviously there are decided limitations in the conclusions to be drawn from a biometrical study of so few strains but an investigation of the characteristics on which species differentiation has been based has been of very considerable interest.

The following characteristics of these forty-four cultures have been studied in this work: (1) morphology, including a study of form, arrangement, capsule formation on different media, and motility; (2) staining reactions, and here the relation of this group to the bactericidal or bacteriostatic effect of gentian violet has been investigated, as well as the gram-staining reaction of the group; (3) cul-

5. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1890, 23, p. 380.

6. *Ibid.*, 1902, 39, p. 1.

7. *Jour. Infect. Dis.*, 1904, 1, p. 241.

8. *Centralbl. f. Bakteriolog.*, Abt. 1, Orig., 1889, 25, p. 49.

9. *Ibid.*, 1912, 66, p. 110.

tural features and biochemical reactions including also a study of the question of the production of a diffusible toxin by certain of the gram-negative, encapsulated bacilli; (4) the immunity or specific serum reactions; and (5) the pathogenicity.

It is hardly to be expected that results of very great value from the point of view of differentiation were to be gained by a study of form alone. An analysis of the results of smears examined shows that the members of this group vary in form from cocco-bacilli to long rods not the least suggesting the coccus-like forms. The length of the bacilli seen in different cultures varied greatly, and cultures with the same designation, from different sources, showed extremely wide variations in morphology. This was perhaps the most conspicuous feature. The cocco-bacilli were found amongst cultures of the so-called bacilli rhinoscleromatis, pneumoniae Friedländer, and capsulatus mucosus. There was no uniformity in this, however, and curves do not show any characteristic mode for any group of these cultures. The arrangement of the bacilli did not tend to show any constant feature, such as the parallel arrangement seen in cultures of the diphtheria bacillus. All the cultures here included presented capsules at some time or other. A few had lost the capsule between the time of isolation and the beginning of this work. Some of these cultures were made to regain their capsule by passage through animals.

All studies of morphology and capsule formation were undertaken on agar cultures after twenty-four hours at 37 C., for form, and on agar and blood serum cultures, for capsule formation. The methods of Hiss, Rosenow and Buerger for demonstrating capsules were utilized. All of these methods gave very satisfactory results. All micro-organisms which were included in this group were encapsulated bacilli or cocco-bacilli, though the facility with which the capsule is lost varies greatly in different cultures. So far as the characteristics already studied are concerned, all of these micro-organisms could very well be included under one species. None of the forty-four cultures here studied was motile.

The staining reactions were next analyzed. Though the point was made by Fricke, and by later workers, including Perkins, Strong and others, the Gram staining characteristics of these bacteria are still used to distinguish the ozena bacillus and the rhinoscleroma bacillus, as I have elsewhere pointed out. McFarland¹⁰ in his text-book, and

Page¹¹ in an article on the ozena bacillus, state that the rhinoscleroma bacillus is a gram-positive bacillus, and so can easily be differentiated from the ozena bacillus.

We have yet to find a culture, in this group, when stained by Gram's method showing the characteristics of a gram-positive micro-organism, and it is probable that, unless one is dealing with a gram-negative encapsulated bacillus, it does not belong to this group. This point cannot be too strongly insisted on as the present great confusion, with reference to the group under discussion, is in part a result of species differentiation based on insufficient data. With the study of the gram-staining properties of the group, the influence in the growth of the cultures of solution of gentian violet on agar, and on serum-agar plate cultures, was observed. Here again, the result was interesting. Of 3 cultures of the ozena bacillus, the growth of 1 was influenced, while 2 were not affected; with 5 cultures of the bacillus pneumoniae, 2 were inhibited, but 3 were not. Five of the 14 cultures of the bacillus capsulatus were inhibited, and 9 were not. None of the rest of the 40 cultures were in any way modified in their growth in the presence of gentian violet solutions. In the three species, members of which were inhibited, there was no constancy shown, and further from 33.33 + to 40 percent of each group were so affected. Although the action of gentian violet, in inhibiting the growth of bacteria, has served to differentiate certain other closely related species it was of no value in this instance. In connection with this interesting action of gentian violet first described by Churchman,¹² we have found it to be of value in the isolation of gram-negative encapsulated bacilli. These micro-organisms, when found in certain situations, especially in the nose, are commonly found associated with various species of gram-positive cocci which tend to overgrow at first, the more delicate gram-negative bacilli. By the use of gentian violet serum-agar plates, we have been able to isolate these bacilli much more readily than by using the ordinary plating methods.

In studies of the cultural features of bacteria, much stress is often laid on differences of minor importance which really are of little value in differentiation. On agar slants, potato, and other media, members of the capsulatus mucosus group are believed to present a slimy mucoid growth. This is most variable. If the culture medium

11. *Jour. Med. Research*, 1912, 26, p. 489.

12. *Jour. Exper. Med.*, 1912, 16, p. 221.

is freshly prepared and contains an abundance of moisture this characteristic is in evidence, otherwise one does not frequently observe it.

The cultural characteristics on potato medium, agar, blood serum in broth, etc., did not present anything of value in differentiation. No previous workers had investigated a large series of these cultures to determine whether their biochemical activities (other than their action on carbohydrates) might be of value in showing differences in species, consequently we devoted considerable attention to this question. We have found that strains of the bacilli capsulatus mucosus, ozenae and pneumoniae Friedländer, all may show proteolytic enzyme activity, as evidenced in their ability to digest blood-serum. Here again, though nothing was learned which could be used to differentiate the species. Similarly the enzyme activity of various members of the group was studied in gelatin. Three cultures of the bacillus capsulatus mucosus and one of the bacillus rhinoscleromatis gave most marked evidence of ability to liquefy gelatin. Cultures which gave parallel results in gelatin were found, however, to differ in many essentials in their behavior on carbohydrates, so that their action on gelatin was apparently not a characteristic which could be utilized to arrange species groups. Only about 10 percent of the cultures of this group liquefied gelatin.

Twenty-three of forty-four cultures were shown to be capable of reducing nitrates to nitrites. Sulphanilic acid was used. Here again, there was no constant reaction according to designation of the cultures. Seventy-five percent of all the cultures were able to produce ammonia when tested with Nessler's reagent, but all species showed about an equal ability in this direction. In a series of tests of the Vosges-Proskauer reaction, using tubes of 1 percent glucose peptone water, and later testing by adding potassium hydroxid solution, 8 of 43 cultures gave a positive reaction. Of the 8, 3 were labelled *B. capsulatus mucosus*, 3 *B. capsulatus* Pfeiffer, and 2 *B. aërogenes*.

All cultures were tested for indol production and all but eight gave evidence of ability to form this substance from the peptone molecule.

Here again, the cultures which did not form indol could not be grouped together when their action on carbohydrates was investigated, as no relation between these characteristics was found. Constant endeavor was made to parallel findings with any relations shown to exist as a result of the study of the fermentation reactions, because all the groupings so far suggested, with the exception of Beham's,

are based either on the action on carbohydrates, or carbohydrates and milk media.

Gèrmain claimed to have been able to differentiate closely related species by means of their ability to produce creatinin. Folin's and Solkowski's methods were employed for this purpose, and all cultures were tested for creatinin production. In all, eight cultures were shown to produce creatinin in 20 percent Dunham's solution after seven days, when tested by the colorimetric method using picric acid and 10 percent sodium hydrate and potassium bichromate solution as a standard, and also by using Weyl's sodium nitro-prusside method. Thus, while it was shown that various gram-negative encapsulated bacilli could produce creatinin, it was not possible to adduce evidence, which with other points elucidated, would serve to show that more than one species were concerned. As we analyze the evidence, on which earlier workers based their claim that we have several species amongst the gram-negative encapsulated bacilli, the results of the activity of our cultures in litmus milk are most instructive. Fricke found coagulation of milk with cultures of the *bacillus pneumoniae* Friedländer; Clairmont, Strong, and Perkins found no coagulation. In repeated tests with cultures of the *bacillus pneumoniae* Friedländer, from two different sources, one from the Pasteur Institute, Paris, and one from the American Museum of Natural History, one of these always failed to coagulate milk, while the other invariably produced coagulation. These results were obtained with chemically clean glassware. It would seem that any classification which emphasizes the point of differences in litmus milk, as an aid to the differentiation of species must be accepted with caution. Perkins has shown also that a culture of the *bacillus capsulatus mucosus*, which originally coagulated milk, did not do so when investigated by Strong and by himself. Furthermore, recent work has shown the fallacy of relying too much on fermentation tests since bacteria can be greatly modified in this particular, as Penfold has emphasized. Changes in litmus milk, ending in acid production and coagulation, are probably the result of lactose being broken up, consequently this test has about the same value as one of the fermentation tests.

These results with the *bacillus pneumoniae* Friedländer, so far as they relate to Strong's work and conclusions, are paralleled by findings with other members of the group, for example, with the *bacillus rhinoscleromatis*, where the same variation is found.

The next question, with reference to the classification of the members of this group, is the significance of their reactions in carbohydrate media. A classification based on one feature alone is of value when constant findings can be obtained. Obviously, if we are sure that all gram-negative encapsulated bacilli can be classified according to their biochemical reactions in carbohydrate media, the problem is greatly simplified. Therefore, we have endeavored to determine whether the fermentation tests may be regarded as satisfying the claims made for them. Strong in his work emphasized the value of three sugars; one monosaccharid-glucose, and two disaccharids—saccharose and lactose. Perkins utilized the following: the monosaccharids—dextrose and levulose; the disaccharids—lactose, maltose, and saccharose, the pentose arabinose, the triatomic alcohol glycerin and the hexatomic alcohol mannite, eight carbohydrates in all. In this work we increased the number of carbohydrates and alcohols to seventeen and we also used the protein sodium caseinate or nutrose.

We have utilized the monosaccharids—glucose, levulose, and galactose; the disaccharids—lactose, maltose, saccharose; the trisaccharids—raffinose; the polysaccharids—dextrin and inulin, the glucoside salicin, the pentoses arabinose and rhamnose, the hexahydric alcohols mannite, sorbite and dulcite, the pentahydric alcohol adonite, the trihydric alcohol glycerin, and the protein sodium caseinate. The method of procedure has been as follows: Each micro-organism has been tested to determine whether or not it would ferment each of these c.p. carbohydrates, alcohols, and the protein, using litmus and phenolphthalein as indicators. With the latter the change in the reaction of the given medium could be determined, in each instance, by using an uninoculated tube as a control. With both indicators any evidence of gas formation in the fermentation tubes was recorded. The sugar broths were prepared according to the standard methods of the laboratory section of the American Public Health Association, using 1 percent of each substance. It may be said at once that nutrose (sodium caseinate) has been of no value whatever, and no further reference need be made to this substance here.

Titration were made after four days at 37 C., with phenolphthalein as indicator; parallel observations were made at the same time with litmus as indicator and any evidence of gas formation recorded.

An analysis of these fermentation reactions shows that it is quite impossible to divide this group into species by means of differences shown in the reaction in the various carbohydrates and alcohols. Table 1 shows that in each instance some of the members of each group ferment one or more of the carbohydrates and alcohols of the different classes. Furthermore it will be observed that the more complex carbohydrate molecule is just as likely to be attacked as is a simpler one. This is not in accord with the findings of Howe,¹³ Winslow¹⁴ and others who have made biometrical studies of other groups of species. Where there is an increased acidity, with none in the control, or where blue litmus has changed to red, or where gas has been observed it is taken for granted that the carbohydrate has been fermented or split up. We believe that the present division of encapsulated gram-negative bacilli into various species here enumerated is not justified on the basis of their fermentation reactions. In connection with the fermentation reactions we have found eight members of this group of fourteen investigated, including *Bacillus capsulatus* Pfeiffer, *Bacillus rhinoscleromatis*, *Bacillus pneumoniae* Friedländer and *Bacillus sputigenus*, all of which give gas formation in lactose bile. As this medium is used as a presumptive test for the presence of the *Bacillus coli* in the examination of water samples, where sewage pollution is suspected, these results are of interest as they show that the statement of the committee of the Laboratory Section of the American Public Health Association, 1912, may require revision.

We have been able in this work to show in three experiments the presence of something in the Berkefeld filtrate of broth cultures of the *Bacillus capsulatus mucosus* which, injected intravenously into guinea-pigs, caused death in less than twenty-four hours. This may be a diffusible or soluble toxin since the heart's blood culture in each instance was quite sterile, as was also the Berkefeld filtrate. This finding, which is in accord with that of Babes¹⁵ and of Pawlowsky,¹⁶ with the *rhinoscleroma* bacillus has no value in the differentiation of members of this group.

Our results with reference to the pathogenicity of the members of this group are in accord with those of Perkins, and are not satisfactory as a basis for classification. We have used guinea-pigs and rabbits and have found that cultures designated as *B. rhinoscleromatis*,

13. *Science*, N. S., 1912, 35, p. 225.

14. *Jour. Infect. Dis.*, 1912, 10, p. 285.

15. *Handb. d. path. Mikroorg.*, 1913, 3, p. 436.

16. *Deutsch. med. Wchnschr.*, 1894, 14, p. 303.

TABLE 1
FERMENTATION REACTIONS OF THE MUCOSUS CAPSULATUS GROUP

	14 Cultures <i>B. capsulatus mucosus</i>	7 Cultures <i>B. pneumoniae</i>	5 Cultures <i>B. rhinoscleromatis</i>	5 Cultures <i>B. capsulatus Pfeiffer</i>	6 Cultures <i>B. aerogenes</i>	4 Cultures <i>B. ozenae</i>	1 Culture <i>crassus sputigenus</i>
Arabinose	11 of 14 +	3 of 4 +	4 of 5 +	4 of 4 +	4 of 4 +	3 of 4 +	1 +
Rhamnose	11 of 14 +	3 of 4 +	4 of 5 +	4 of 4 +	4 of 4 +	2 of 4 +	1 +
Dextrose	14 of 14 +	7 of 7 +	5 or 5 +	5 of 5 +	6 of 6 +	4 of 4 +	1 +
Galactose	13 of 14 +	4 of 4 +	5 or 5 +	4 of 4 +	4 of 4 +	3 of 4 +	1 +
Levulose	14 of 14 +	3 of 3 +	5 or 5 +	4 of 4 +	3 of 4 +	4 of 4 +	1 +
Lactose	13 of 14 +	6 of 7 +	5 or 5 +	5 of 5 +	6 of 6 +	3 of 4 +	1 +
Maltose	14 of 14 +	5 of 7 +	5 or 5 +	5 of 5 +	5 of 6 +	2 of 4 +	1 +
Saccharose	12 of 14 +	7 of 7 +	3 or 5 +	5 of 5 +	6 of 6 +	2 of 4 +	1 +
Dextrin	12 of 14 +	5 of 7 +	3 or 5 +	4 of 5 +	5 of 6 +	2 of 4 +	1 +
Inulin	8 of 14 +	4 of 7 +	3 of 5 +	5 of 5 +	5 of 6 +	1 of 4 +	1 +
Raffinose	11 of 14 +	5 of 7 +	3 of 5 +	5 of 5 +	6 of 6 +	2 of 4 +	1 +
Adonite	11 of 14 +	4 of 5 +	3 of 5 +	5 of 5 +	5 of 6 +	3 of 4 +	1 +
Dulcitol	All negative	2 of 7 +	3 of 5 +	5 all negative	2 of 6 +	All negative	1 negative
Naumite	13 of 14 +	6 of 7 +	4 of 5 +	5 of 5 +	5 of 6 +	2 of 4 +	1 +
Sorbitol	13 of 14 +	9 of 9 +	5 of 5 +	4 of 4 +	4 of 5 +	3 of 4 +	1 +
Glycerin	13 of 14 +	3 of 4 +	5 of 5 +	4 of 4 +	5 of 6 +	2 of 4 +	1 +
Salicin	9 of 14 +	5 of 7 +	5 of 5 +	5 of 5 +	6 of 6 +	2 of 4 +	1 +

+ = splitting of carbohydrate with acid or gas production.

B. capsulatus mucosus, *B. pneumoniae* Friedländer, etc., gave similar results when intraperitoneal injections were given. Some of the cultures had lost their virulence, but this was not characteristic of any one group.

The agglutination reaction and the reaction of fixation were employed in an endeavor to find a satisfactory basis of classification with results, which so far, are of limited value.

Beham and we have shown that members of this group are not agglutinable until they have lost their capsule. But they may act as antigens and produce agglutinins while still possessing a capsule. After they have lost their capsules they are agglutinable by appropriate sera. So far our results may be summarized as follows:

Cultures from various sources designated as *B. rhinoscleromatis* were agglutinated with immune rabbit serum. Unfortunately cultures with other designations possessed capsules while those of the cultures of the rhinoscleroma bacillus have been lost, so that differentiation was impossible. Further work in this direction will be undertaken. It may be said at this juncture, however, that if only those strains which have lost their capsules are agglutinable, it is obvious that with the great majority of freshly isolated cultures the reaction of agglutination will have no value as a means of differentiation. The acid agglutination reaction of Michaelis as modified by Beniasch¹⁷ did not cause agglutination of the bacterium of rhinoscleroma without a capsule, or bacillus mucosus with a capsule.

The reaction of fixation has been tried with indifferent success. Antigens employed were first, broth cultures of the different strains but later antigens prepared according to the method used by Claypole¹⁸ in her work with the streptothrix group was also tried. Rabbits were immunized with different strains and their sera with the different antigens and guinea-pig alexin was used. Cross fixations occurred even with quite low dilutions and very considerable anticomplementary activity was observed. While satisfactory fixation reactions were obtained, the homologous antigens and antisera gave no more satisfactory results than the heterologous. We were unable to obtain satisfactory results in the differentiation of Friedländer bacillus and the rhinoscleroma bacillus, by means of the reaction of fixation. We have so far been unable to obtain similar result.

17. *Ztschr. f. Immunitätsf.*, 1912, 12, p. 268.

18. *Jour. Exper. Med.*, 1913, 17, p. 99.

SUMMARY

So far our work cannot be taken to support any grouping of the gram-negative encapsulated bacilli heretofore proposed. Such divisions have been based on differences in staining reactions, cultural features, biochemical activities, or pathogenicity. After a careful review of these points in the cultures studied by us, it does not seem possible, at the present time, to constitute species on the basis of differences shown. It seems more than likely that this group is most closely related to the colon, the essential point of distinction being the possession of a capsule. It is conceivable that mutations, based on the necessity of maintaining a parasitic existence, have caused gram-negative bacilli, found normally in the body elsewhere than in the intestinal tract, to develop capsules for protection and a new group has arisen which we designate *B. capsulatus mucosus* and the varieties *B. aërogenes* and *B. acidi lactici* connect the group with the non-encapsulated gram-negative bacilli belonging to the colon group. This is not offered as a conclusion arising from the results here presented, but rather as a tentative suggestion.

THE FORMATION OF ANTIBODIES IN RATS FED ON PURE VEGETABLE PROTEINS (OSBORNE- MENDEL STUNTING FOOD) *

LUDVIG HEKTOEN

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Osborne and Mendel have made the important discovery that by feeding white rats certain pure vegetable proteins normal growth is arrested without disturbance of the general health, the animals resuming growth on the resumption of ordinary food or when certain amino acids are furnished.¹ As I was engaged in a study of the formation of antibodies under different conditions, it occurred to me that it would be of interest to try to determine whether or not the formation of antibodies proceeds in the usual way in rats whose growth is arrested by this means. Accordingly, the amount of specific lysin for sheep corpuscles, produced in normal and stunted animals under comparable conditions, has been determined.

The blood-serum of white rats normally causes lysis of sheep corpuscles. This action is dependent on an amboceptor-complement mechanism, because on being heated at 56 C. for thirty minutes the serum loses its lytic power, which is restored, however, on the addition of complement in the form of fresh guinea-pig serum, in quantities that in themselves do not cause laking of sheep corpuscles. The amount of amboceptor in normal rat serum varies, but usually no lytic action is obtained in dilutions of the serum above 1 to 96. When white rats are injected with defibrinated sheep blood, there is produced a specific lysin for sheep corpuscles which follows a course, in general, like that of other antibodies under similar conditions. Two methods of injection have been tried: a single intramuscular injection of 1 c.c. of a 10 per cent. suspension of sheep blood per kilo of weight; and a single intraperitoneal injection of 1 c.c. of a 10 per cent. suspension per kilo. The amount of lysin produced is much greater in the case of the second method, which is the method used in these experiments. In either case

* Received for publication, May 19, 1914.

1. Beobachtungen über Wachstum bei Fütterungsversuchen mit isolierten Nahrungssubstanzen. Hoppe-Seyler's Ztschr. f. physiol. Chem., 1912, 80, p. 307; Amino-Acids in Nutrition and Growth, Jour. Biol. Chem., 1914, 17, p. 325.

the high point in the new production of lysin is achieved early, about the fifth or sixth day and occasionally even earlier, and by the tenth or eleventh day the concentration begins to lessen, the normal level being reached or closely approached in from thirty to forty days after the injection. In other animals, injected with a single dose of antigen, the climax in the production of antibody usually does not occur until a few days later than is the case in the white rat after the injection of sheep blood.

On account of the small size of the rat it is necessary to bleed from the heart in order to obtain enough serum. This is not difficult until adhesions form in consequence of the repeated punctures; however, quite a few animals die from hemorrhages into the pericardium. The possible effect of the loss of blood must not be overlooked; in animals weighing 75 to 150 gm. the withdrawal of 0.3 to 0.5 c.c. of blood is a serious loss, and as loss of blood is believed to stimulate the production of antibodies in other animals, it is highly probable that in the rats of these experiments the production of lysin was stimulated by the bleedings. In judging the results, it must be borne in mind that a complicating factor of this kind no doubt may have a varying effect in different animals.

In order to learn the effects of the Osborne-Mendel food on antibody production, two sets of experiments have been carried out, each involving a group of stunted and of normal rats. Similar results were obtained in each case and the essential points are illustrated in Charts 1 and 2, which give the weight curves and the antibody curves of one set. The stunted rats were placed on the special food five days before the injection of the sheep blood, so that the first day of Chart 2 corresponds to the sixth day of Chart 1. The materials for the food were supplied by Professor Mendel, to whom I am greatly indebted, and the food was prepared according to his directions, as follows: take 380 gm. corn gluten (specially prepared), 280 gm. "protein-free milk," 20 gm. Duryea's corn starch, 320 gm. lard; melt the lard at a low temperature and stir into it the other ingredients previously mixed; cool after thoroughly mixing, and put through an absolutely clean meat chopper several times. The paste keeps well in glass jars. The rats were kept supplied constantly with all they would eat, care being taken to pack the food well in suitable cups. The cages were kept clean and fresh water supplied regularly. That this food arrests growth is shown at a glance by Chart 1. The control animals were fed as usual.

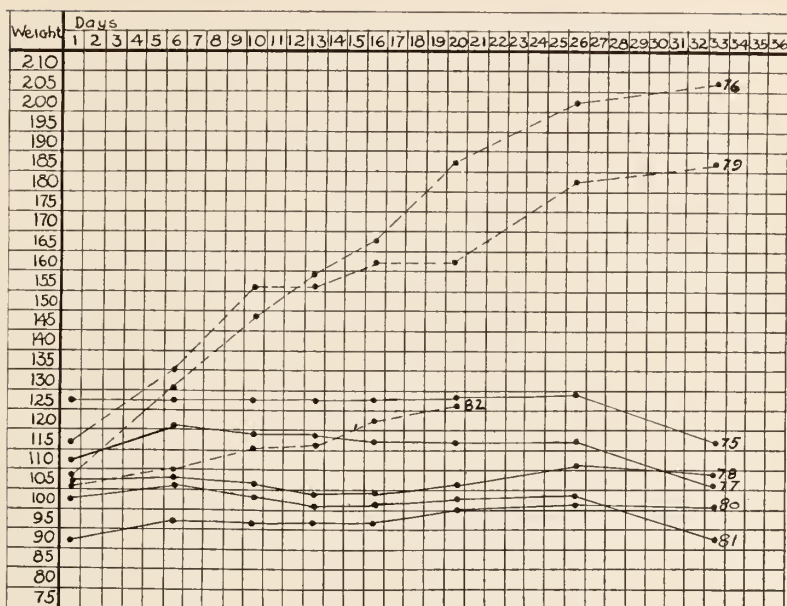


CHART 1.—The Weight Curves. The solid lines indicate the weights of rats on the Osborne-Mendel food; the broken lines the weights of rats on ordinary food.

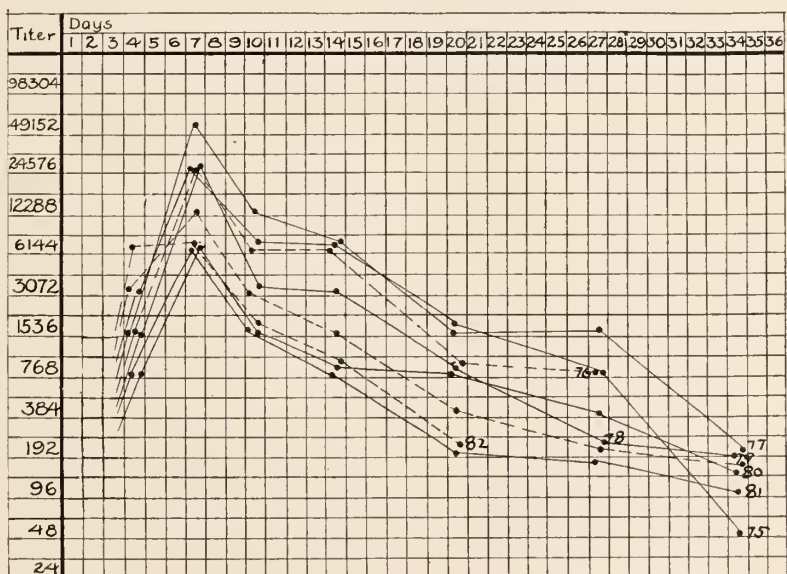


CHART 2.—Antibody (Lysin) Curves. The solid lines refer to rats on the Osborne-Mendel food, the broken lines to rats on ordinary food. Day 1 on this chart corresponds to the sixth day of Chart 1.

In order to reduce the chances of accidental death the first bleeding was made on the fourth day after the injection of the antigen (1 c.c. of a 10 percent suspension of sheep blood per kilo of weight intraperitoneally), then every third day two times, and after that at longer intervals. The determinations of the lysin were made by finding the highest dilution of the serum, heated at 56 C. for thirty minutes, and complemented with guinea-pig serum (.0125 c.c.), at which distinct lysis was produced. The total quantity in each tube was 0.6 c.c., including 0.2 c.c. of a 5 percent suspension of sheep corpuscles, carefully washed, .0125 c.c. of guinea-pig serum, and the requisite amount of heated rat serum, the rest being salt solution. The mixtures were incubated for two hours and then placed in the ice-box until the following morning.

The results require no comment (Chart 2). The curves run fairly parallel; there is no outstanding difference in the results in the stunted and the normal rats, and there is just as much variation between the individual members of one group as of the other. That there should be variation is not surprising especially in view of the probable influence of the bleedings, to which attention has been called.

It appears then that, so far as the results of these experiments indicate, the arrest of growth in rats by the Osborne-Mendel method does not cause any disturbances in the production of antibodies.

THE BACTERIOLOGY OF VAGINITIS*

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In the treatment and management of vaginitis in little girls there is a certain amount of confusion which arises from the uncertainty regarding etiology. Clinically, the affection resembles gonorrhea, and occasionally stained smears show typical gonococci. Certain cases are of known gonococcal origin, and many more are generally regarded as gonorrheal. The majority of cases, however, do not give the typical gonococcus picture with intracellular cocci. A positive gonococcal diagnosis from vaginitis smears is frequently based merely on the presence of forms which may actually be common pus cocci or even coccoid bacilli (colon bacilli). Such an error might be easily made from methylene blue stains and even from gram-stained smears if coccoid forms of the colon bacillus or if degenerated gram-negative staphylococci are present.

The question arises whether all these cases are primarily gonococcal. Even discharges of known gonococcal origin often continue, become less profuse, and of a serous character unlike ordinary gonorrhea. In such cases it is often assumed that the gonococcus has been replaced by other organisms, which have become parasitic in the less resistant vaginal mucosa following a primary gonorrhea.

The object of this work is to determine as far as possible whether vaginitis in children is gonococcal, and if so how long the gonococcus continues to be the specific exciting cause. My work consists in the bacterial examination of vaginitis by cultural and immunological methods, with special reference to the rôle played by the gonococcus. With this in view, the hypersensitiveness to and the immunity against known gonococcal products have been used in connection with the work.

The paper deals with a certain class of cases in the vaginitis wards of Chicago hospitals, with vaginal discharges diagnosed as gonococcal from the hospital smears. The patients varied in age from 15 months to 13 years and were largely from the poorer classes. Vaginitis was observed in four cases following rape and probably gonorrheal, in

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eighteen cases without known cause, and in five accompanying or following the exanthemata. The discharges varied in duration and amount, and for the most part had resisted treatment. Very few showed typical gonococcus forms by gram-stained and carefully examined smear. Vaginitis in such a class of cases is more suspicious of gonorrhea than in certain other classes, which are not to be considered here.

The complete aerobic bacteriology of fifteen of these cases was studied. The discharge was smeared over a hard human blood agar plate with one applicator, and then spread thinly with a second. The plates were examined in twenty-four and forty-eight hours, and colonies transferred to blood agar slants, and later identified culturally and microscopically. Aside from the gonococcus, the commonest were the staphylococcus, the colon bacillus, and the pseudodiphthericus bacillus. The latter, referred to as *B. pseudodiphthericus*, is a gram-positive bacillus, occasionally pleomorphic, which forms small transparent colonies on agar and blood agar, does not ferment dextrose, and some strains slightly alkalinize milk. It evidently corresponds to the organism designated by this name by other workers on vaginitis.

From one of the fifteen cases all three bacteria, the staphylococcus, the colon and pseudodiphthericus bacilli were isolated; from six, the pseudodiphthericus and the staphylococcus; from four, the pseudodiphthericus and the colon bacilli; from three, the staphylococcus; and from one, the colon bacillus without the others. Hemolytic strains of both the staphylococcus and the colon bacilli were the more common, but in a majority of cases both hemolytic and non-hemolytic strains of each were present. Other bacteria, chiefly from the streptococcus, proteus and acid-fast groups, were present in one, two or three cases. It is probable, as Curtis¹ points out, with reference to leukorrhea in women, that these aerobes are present only in the vulvar region unless frequent douching or other manipulation carries them deeper, where the vagina is otherwise infected only by anaerobes.

Five cases, with normal vaginas, were examined and the same three organisms, staphylococcus, colon bacillus and pseudodiphthericus bacillus were isolated; however, this number of cases is too small to give any idea of the frequency of their occurrence. Küster,² reviewing the work done on normal vaginal flora, shows the predominance

1. *Surg., Gynec. and Obst.*, 1914, 18, p. 299.

2. *Handb. d. path. Microorg.*, 1913, 6, p. 458.

of anaerobic organisms and points out that the pyogenic bacteria are present in but a small percentage of normal cases. Whether the larger proportion of our vaginitis cases, found to harbor these organisms, means that these bacteria are associated with the pathologic process, is a question. That they are not the sole exciting factor, and that they do not entirely replace the gonococcus, appears from the following.

Examinations for the presence of gonococci were also made by means of human blood agar plates. For this purpose the plates were incubated in a moist chamber at a temperature between 35 and 37 C. An ordinary desiccator jar, containing the plates over a little water and kept in the thermostat, was used successfully. After twenty-four hours, ten of the colonies resembling gonococcus were transferred to blood agar slants, and this was repeated after forty-eight, and sometimes seventy-two and ninety-six hours. The resulting growths were examined and identified. Gram stains were made of the same discharge, and records kept of the history and appearance of the same.

The isolation of the gonococcus was possible only by observing the precautions given above, and even then, in some cases, two, three, or even four repetitions of the work were necessary before the organism was isolated. From this, it seems probable that the gonococcus was present in even a larger number of cases than we found it. Similar examination of normal vaginal secretions revealed no organism resembling the gonococcus.

In the bacterial examinations of vaginitis discharges, twenty-seven were under cultural conditions favoring the growth of the gonococcus. After one or more attempts, gram-negative diplococci, which did not grow on plain agar, presumably the gonococcus, were isolated from twenty-two of the twenty-seven, though only fifteen of these strains could be kept in stock long enough for confirmation of their identity. These fifteen differed slightly from other stock gonococcal strains in that they seemed more susceptible to slight loss of moisture, though several were less susceptible to a rise of temperature to 42 C., for several hours. According to most authorities, reviewed by Koch,³ the gonococcus is killed in a few hours by a temperature of 40 to 41 C., though one, Wertheim, found that it could live at 42 C. Of all our gonococcal strains, none withstood this temperature save some of those isolated from vaginitis. They grew more scantily on media than most

3. Handb. d. path. Microorg., 1912, 4, p. 684.

strains from other lesions, but grew to some extent on plain agar after fewer transfers, some after only three. Koch finds that four or five generations on humanized media are usually necessary before growth on plain agar is possible.

These variations called for further assurance that they were strains of gonococcus, though some were from vaginitis cases almost surely of gonococcal origin. Thus, for example, Case 12, 13 years old, was admitted for vaginitis following alleged rape. There was a profuse discharge and microscopic examination showed it to be probably gonococcal. The organism was isolated on the first attempt. This strain, almost surely gonococcus, showed the same variations in susceptibility as those noted above. To confirm the identity of these several strains the complement-fixation test was used. Thick suspensions of the doubtful strains were made, heated to 60 C. for an hour, and used as antigen together with serum which had fixed complement with known gonococcus antigen. They all fixed complement, and were hence diagnosed certainly as gonococcus.

The number of gonococci which developed from the different discharges varied greatly. With approximately the same amount of material spread on each plate, some produced very many gonococcus-like colonies to the plate, others very few. There was no relation between the amount of discharge in a given case and the number of gonococci developing from the amount plated. From ten of the discharges there were a large number of gonococcus-like colonies to the plate, and of these ten, four were abundant, or moderate, and six scant. Of the other discharges, producing very few gonococcus colonies to the plate, ten were abundant and nine scant. From this it appears that the amount of discharge bears no relation to the number of gonococci present. A majority had but a slight discharge when examined, and in one the discharge had completely stopped. In the latter case the patient had been pronounced cured, and was about to be removed to a non-vaginitis ward when the gonococcus was first isolated.

The presence of gonococci after cessation of the discharge suggests that the original infection is responsible for the recurrences so frequently observed. Spalding⁴ reported twenty-six non-selected cases which she had been able to trace, and found that all but two had one or more recurrences of the discharge at intervals of three weeks to

4. *Am. Jour. Dis. Child.*, 1913, 5, p. 248.

six years. Others⁵ have thought it improbable that the gonococcal infection remains latent, and that a better explanation of these recurrences is reinfection with gonococcus, or infection with other organisms which find the mucosa a favorable ground for development due to the previous infection. From our results, latent infection would seem to be a probable factor.

Besides the cultural examinations, work was done to determine whether or not the gonococcus had excited the production of antibodies in these cases, and whether or not immunity reactions could be depended on as diagnostic of the presence of the gonococcus. The hypersensitiveness to known gonococcus toxins was tested by the skin reaction.⁶ Glycerin extracts of gonococcal protein introduced on a needle point at two places in the skin, caused zones of hyperemia which were not produced by two other control punctures. These were measured after twenty-four hours and their size noted and averaged. In gonococcal cases a large zone appears when the allergic curve is high, but a smaller zone or none at all when it is low. In non-gonococcal cases there may also be a small zone due to non-specific irritation of the toxin, especially to a very sensitive skin, or to anaphylactic bodies normally present. However, it seemed safe to consider cases positive (gonococcal) if the two zones averaged 5 mm. or together with a marked papule 4 mm., doubtful where they averaged 2 to 4 mm., and negative if less than 2 mm.

The power of the patient's serum to fix complement was also tested, together with antigen, consisting of known gonococcal suspension heated to 60 C. for an hour. If non-gonococcal, the serum is expected to give a negative test, though Smith⁵ finds that it may at times be weakly positive. The serum of a gonococcal case may be positive or negative depending on whether the immune curve is high or low when the blood is drawn. In our cases this curve apparently paralleled that of the cutaneous reactivity, for nine cases with positive skin tests also had sera which fixed complement, and two of the nine at another time did not give a positive skin reaction, nor a positive fixation test. A tenth case with a positive skin test was not tested for complement-fixation. The sera from cases giving a doubtful or negative skin test did not in any case fix complement.

5. Smith, *Am. Jour. Dis. Child.*, 1914, 7, p. 230.

6. Irons, *Jour. Infect. Dis.*, 1912, 11, p. 77.

The nature of this immune curve is not shown by our work, as this would involve frequent tests on an individual case. From the table following, however, it will be seen that most of the cases giving positive tests had discharges of less than three months' standing, while the negative results are largely from older cases. This would suggest that the curve is high in an early infection only, and falls after a few months. Naturally, the discharge also becomes less after a few months of treatment, but the immunity curve's decline did not depend on the decrease of the discharge apparently, for the latter was often quite active when the tests were negative.

The typical case seemed to be with a high immunity curve early. This soon decreases, followed later by a decrease in the discharge, and still later, if at all, by the elimination of the gonococcus. With Case 3, after seventeen days, both cutaneous and complement-fixation tests were positive and the discharge profuse. The discharge remained profuse for several weeks though the complement-fixation test became negative two weeks after the first test and remained negative, the cutaneous test being then doubtful. Case 22 was first examined more than four months after the beginning of the discharge. The cutaneous test was doubtful, each zone being red over 2 mm. diameter and red-dish over 4 mm. Several days later it was found to be fainter and after a month was quite negative, though there was still a scant discharge. Two months after this it was still negative, and the discharge had completely stopped, but at this time the gonococcus was isolated, and with known gonococcal serum it fixed complement. These seem to be typical cases. Only one case suggested that the curve may not follow this even decline. Case 24 still had a discharge of more than a month's duration when neither complement-fixation nor cutaneous tests were positive. Two weeks later, both were markedly positive, the discharge remaining about the same. From this case the gonococcus was not isolated. Here, the curve was late in reaching its height or was temporarily low when first tested.

Determinations made from stained smears seem to have no more constant value than those made from the immunity reactions. After Gram staining and careful examination of smears of forty-one discharge specimens, only seven were found to be clearly gonococcal, fourteen probably so, and twenty too doubtful for diagnosis. And yet almost all these cases were found to be gonococcal. Even a Gram stained smear can easily seem to be gonococcal, due to the failure of

the staphylococcus to hold the stain, as sometimes occurs, and to coccoid forms of gram-negative bacilli. Pus cells in these cases are usually few, and intracellular cocci much fewer, and so it is hard to be sure whether a slide is or is not gonococcal. The seven slides, called definitely positive, were those which contained intracellular gram-negative diplococci or extracellular gram-negative diplococci without the presence of rod forms. It is only such slides that we could depend on for proof of the presence of gonococci.

The definitely positive smears are chiefly of discharge from which large numbers of gonococcus colonies developed on the plates. On the ten examinations, in which very many gonococci were thus shown to be present in the discharge, only two of the stained smears were doubtful, four being definitely positive, and four probably positive. The large percentage of doubtful smears were principally from discharges containing relatively few gonococci. These points suggest that stained smears do not indicate the presence or absence of gonococci, but whether or not they are present in very large numbers. Perhaps if enough attempts were made, positive smears could be obtained from discharges having few gonococci; some portions of a discharge would have more organisms than others. Or some examinations might be made at a time of temporary increase in the number present, though we found no evidence of such temporary variations.

Hospital examinations of vaginitis smears usually yield a much larger proportion of gonococcal diagnoses. No criticism of such findings is intended, though many would be listed as doubtful in this report. In hospitals, our doubtful smears might be called positive, to avoid the danger of removing vaginitis cases to non-vaginitis wards. It is also to be borne in mind that these results are from a limited number of smears.

In vaginitis, diagnosis of the infective agent is important, and also the determination of the time of a cure. To determine the infective agent, all of the above mentioned laboratory tests are of value. Nearly half of our cases, on one or more tests, gave a definitely positive cutaneous reaction and nearly all of them a positive or doubtful one. Probably these were all positive at some time during the infection, but as such a time cannot be determined for ordinary diagnosis, the skin test's value is very limited. Neither can it be used to determine the time of clearing up of the infection, for the sensitiveness diminishes and frequently disappears before the infection does. The

same is true of the complement-fixation test, which does not even give a suggestive reaction unless the immunity curve is at its height. A negative complement-fixation test of the patient's serum has been regarded as evidence of cure of vaginitis.⁷ According to our cases it would have no such value, for the gonococcus was isolated from five cases with a negative fixation test at the time, and three of these still had quite an active discharge. The reactivity of the serum seems to decrease and usually to disappear within a few months, whether the gonococcus is still present or not. As slides also cannot be relied on, and as the discharge itself may cease before the infection does, we seem to be without means of diagnosing the cure of vaginitis.

A diagnosis of the infecting organism is made only with difficulty. Of this series of twenty-seven presumably gonorrheal cases, reliable diagnoses could have been made from the repeated cultural tests alone in twenty-two, from complement-fixation tests in nine (of those tested), from cutaneous tests in 10, and from repeated smear examinations in 5. The isolation of the organism would seem from the above to be the most reliable method, though if a case could be tested frequently from the time of infection the immunity tests would be a very great help. In most cases the value of slides was only confirmatory. Combinations of more than one of these methods would point out a large proportion of gonorrheal cases.

Following are lists of the cases on which these conclusions are based. The cutaneous tests are called positive where zones averaging 5 mm. or 4 mm. with marked papule appeared, doubtful where from 2 mm. to 4 mm. and negative where less than 2 mm. The entire series is thought to be gonococcal and from the first twenty-two the gonococcus was isolated. Where more than one examination are noted, the organism was isolated (except from Case 8) in the last.

From this analysis it may be concluded that vaginitis in this series of cases is largely and may be entirely gonococcal in origin, and that the gonococcus is present as long as the discharge continues. If there is a secondary invader it either cannot be easily cultivated aerobically, or it is a normal inhabitant of the vagina, and it does not altogether replace the gonococcus. The immunity to the gonococcus seemed to diminish after a few months, after which the infection frequently persisted. This immunity could be followed by complement-fixation or cutaneous tests. I found the latter more delicate as it gave a

7. Smith, *Am. Jour. Dis Child.*, 1913, 5, p. 313.

TABLE 1

LIST OF CASES FROM WHICH THE *GONOCOCCUS* WAS ISOLATED

Case	Previous Duration of Discharge	Amount of Discharge When Examined	Con.-Like Colonies to Plate	Careful Smear Diagnosis of Discharge	Cutaneous Test	Complement-Fixation Test
1—	? over 3 days	Moderate discharge	Many	Probably positive	Positive	Positive
2—	? several days	Scant discharge	Many	Probably positive	Not tested	Not tested
3—1	17 days	Profuse discharge	Few	Doubtful	Positive	Positive
3—2	2 weeks later	Profuse discharge	Few	Probably positive	Doubtful	Negative
3—3	2 weeks later	Profuse discharge	Few	Doubtful	Not tested	Not tested
3—4	2 weeks later	Profuse discharge	Few	Doubtful	Doubtful	Negative
4—	18 days	Scant discharge	Few	Doubtful	Doubtful (4 mm.)	Negative
5—1	19 days	Profuse discharge	Many	Positive	Doubtful (4 mm.)	Negative
5—2	12 days later	Moderate discharge	?	Positive	Doubtful	Negative
6—	6 weeks later	Scant discharge	Many	Positive	Not tested	Not tested
7—1	2 months	Profuse discharge	Few	Probably positive	Doubtful	Not tested
7—2	1 month	Scant discharge	Few	Probably positive	Positive	Positive
8—1	6 weeks later	Moderate discharge	Many	Probably positive	Positive	Positive
8—2	3 weeks later	Moderate discharge	?	Positive	Positive	Positive
9—	3 months	Scant discharge	Many	Positive	Positive	Positive
10—	? over 3 months	Profuse discharge	Few	Probably positive	Positive	Positive
11—	? over 3 months	Profuse discharge	Few	Probably positive	Positive	Positive
12—	? few months	Profuse discharge	Many	Probably positive	Doubtful	Negative
13—	? few months	Scant discharge	Many	Doubtful	Doubtful (4 mm.)	Positive
14—	4½ months	Scant discharge	Many	Doubtful	Doubtful	Negative
15—	? over 1 month	Scant discharge	Many	Positive	Doubtful	Negative
16—	? recent	Scant discharge	Many	Doubtful	Doubtful	Not tested
17—	? recent	Scant discharge	Many	Doubtful	Doubtful	Not tested
18—	? recent	Profuse discharge	Few	Doubtful	Positive	Not tested
19—1	? unknown	Profuse discharge	Few	Doubtful	Negative	Not tested
20—1	3 months later	Moderate discharge	Few	Doubtful	Negative	Not tested
20—2	? unknown	Profuse discharge	Few	Doubtful	Negative	Not tested
21—1	3 months later	Scant discharge	Few	Doubtful	Negative	Not tested
21—2	? few months	Scant discharge	Few	Doubtful	Negative	Not tested
22—1	2 months later	Scant discharge	Few	Probably positive	Doubtful	Not tested
22—2	Over 4 months	Scant discharge	Few	Doubtful	Doubtful	Not tested
23—	1 week later	Scant discharge	?	Not examined	Doubtful	Not tested
24—1	1 month later	Scant discharge	?	Not examined	Doubtful	Not tested
24—2	3 months later	No discharge	Few	Doubtful	Negative	Not tested

TABLE 2

LIST OF CASES FROM WHICH THE *GONOCOCCUS* WAS NOT ISOLATED

Case	Previous Duration of Discharge	Amount of Discharge When Examined	Careful Smear Diagnosis of Discharge	Cutaneous Test	Complement-Fixation Test
23—	? few weeks	Scant discharge	Probably positive	Positive	Positive
24—1	? few weeks	Profuse discharge	Doubtful	Doubtful	Negative
24—2	2 weeks later	Profuse discharge	Probably positive	Positive	Positive
25—1	3 months	Profuse discharge	Doubtful	Doubtful	Negative
25—2	6 weeks later	Moderate discharge	Doubtful	Doubtful	Negative
26—	? recent	Moderate discharge	Doubtful	Doubtful	Not tested
27—1	? unknown	Scant discharge	Doubtful	Doubtful	Not tested
27—2	3 months later	Moderate discharge	Doubtful	Negative	Not tested

suggestive reaction when the immunity was too weak for complement-fixation. The cases giving a definitely positive test with one did with the other also. Definite diagnoses from smears seemed of value only in the few cases when carefully examined Gram preparations showed the typical gonococcal picture, while a majority of slides could not be called positive unless confirmed by other signs. While laboratory methods are of assistance in determining the nature of the infecting organism, none of them seems to be of value in defining the time of its elimination.

I wish to thank Prof. E. O. Jordan for suggesting this work in partial fulfillment of the requirements for the degree of M.A. at the University of Chicago; Dr. Ernest E. Irons for help throughout the work, and Dr. Homer K. Nicoll, who made the complement-fixation tests.

THE RELATIVE LONGEVITY OF DIFFERENT STREPTOCOCCI AND POSSIBLE ERRORS IN THE ISOLATION AND DIFFERENTIATION OF STREPTOCOCCI *

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In the study of a large number of strains of streptococci I am impressed by the marked variation in the viability of the different strains. I have found that many types of streptococci may be stored for months on various media, while on the other hand, one frequently encounters strains that require fresh food material at short intervals.

The most favorable media for the preservation of the life of streptococci are those in which the organisms do not readily produce self-destructive substances. Such media are plain serum broth, blood agar, gelatin and others. It is very important that the media do not contain available carbohydrates as the acids formed by fermentation are detrimental to the life of these organisms.

EFFECTS OF ACID

Numerous investigators have observed the destructive effect of acids on streptococci. Koch and Pokschischewsky¹ used the fact that the streptococcus equi remained living in mannite serum broth after ten days as evidence that it had not attacked the carbohydrate. They also found that, by neutralizing at intervals dextrose and levulose cultures, acid continued to be produced up to the eighth day.

Wurtz and Mosny,² believing the rapid death of the pneumococcus in their medium was due to the acid produced, added calcium carbonate, and their cultures remained viable from one to six months.

Hiss³ used the same method to obtain material for immunization and agglutination. He used dextrose in his medium and added calcium carbonate to prevent the production of an excess of acid.

In another article I have discussed the relative longevity of different streptococci against the acids produced in fermentation. Koch and Pokschischewsky⁴ demonstrated that the streptococcus longus was more resistant to acid than the

* Received for publication May 29, 1914.

1. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1913, 74, p. 1.

2. Quoted by Neufeld and Händel, *Handb. d. path. Mikroorg.*, 1912, 4, p. 522.

3. *Jour. Exper. Med.*, 1905, 7, p. 223.

4. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1913, 74, p. 1.

streptococcus equi, as the former remained viable on dextrose broth longer than the latter. Von Lingelsheim⁵ has shown that the streptococcus brevis is more resistant than streptococcus longus to hydrochloric, sulphuric and oxalic acids.

EFFECT OF DESICCATION

The effect of drying of the media is important in considering longevity of streptococci. Besides the concentration of the salts and other constituents of the media we have to consider direct action of desiccation. Streptococci vary within wide limits in their power to survive a drying process. The majority of streptococci are very sensitive to desiccation. They usually die in a few days when dried on threads, glass and other substances. Hägler⁶ found that streptococci from erysipelas lived from fourteen to thirty-six days when dried on mull, and Kurth,⁷ that the streptococcus conglomeratus resisted drying for from five to six weeks. Germano⁸ noted that streptococci were very resistant if dried on dust, but different strains showed wide variations in their power to withstand this drying. Andrewes⁹ showed that certain strains, such as streptococcus equinus, will live for months when dried on garnets, while streptococcus pyogenes will only live for weeks under the same conditions.

The pneumococcus is generally much more sensitive to drying than the streptococci. Wood¹⁰ has thoroughly studied the viability of the pneumococcus after drying, and many other investigators have shown that its longevity is relatively short.

I have dried a strain of streptococcus fecalis from dextrose broth on cover-glasses and obtained growth after keeping in diffuse daylight for 174 days. The morphologic, cultural and fermentative characters remained unaltered (Holman).¹¹

The presence of streptococci in the air is an evidence of their resistance to a relatively high degree of drying. Chatin¹² isolated, from the air of rooms, different kinds of streptococci, some of which were highly pathogenic to rabbits, while others were non-pathogenic. Gordon¹³ found several different forms of streptococci in the outdoor air of London, streptococcus brevis being the most common type. Andrewes and Horder¹⁴ have shown that streptococcus equinus is the commonest type present in the air of London. It is also most abundant in horse dung. Rosenow¹⁵ obtained streptococci from the air of the hospital operating-room and the hospital wards. Winslow and Kligler,¹⁶ in a study of

5. Handb. d. path. Mikroorg., 1912, 4, p. 453.

6. Quoted by von Lingelsheim, *Ibid.*

7. *Ibid.*

8. *Ibid.*

9. *Lancet*, 1906, 2, p. 1415.

10. *Jour. Exper. Med.*, 1905, 7, p. 592.

11. *Am. Jour. Pub. Health*, 1913, 3, p. 1210.

12. These-Faculte de Medicine et de Pharmacie de Lyon, 1893, Serie 1, p. 812.

13. *Ann. Rep. Loc. Gov. Bd.*, 1902-3, p. 421.

14. *Lancet*, 1906, 171, p. 708.

15. *Amer. Jour. Obst.*, 1904, 50, p. 762.

16. *Am. Jour. Pub. Health*, 1912, 2, p. 663.

city dust, found in the air a variety of streptococci, chiefly of types characteristic of the human mouth and the human intestines. These authors conclude from their studies that certain streptococci are very resistant to drying.

RESISTANCE TO HEAT

Streptococci are also far more resistant to high temperatures than is commonly supposed. The destructive action of heat on bacteria in general is, after all, a relative one and we find that streptococci exhibit a marked variation in their ability to withstand high temperatures.

Van Lingelsheim¹⁷ has demonstrated that the streptococcus brevis is much more resistant to the action of heat than is the streptococcus longus. The former withstood 65 C. for twenty minutes, 70 for ten minutes, and 80 for five minutes, while the streptococcus longus succumbed after twenty minutes at 55 C., ten minutes at 60, and five minutes at 67.

In a later article the same author says that if a suspension of streptococcus longus is heated in normal salt solution to 60 C. for one or two hours it is not always sufficient to kill all the cocci. One hour at 70 or two hours at 65 is necessary for complete sterilization.

The character and composition of the fluid surrounding the cocci is of the greatest importance in determining the effects of heat. Albuminous fluids, such as milk, blood-serum and ascites fluid, have distinctly protective actions on the contained bacteria. In sterilizing such fluids for use in the preparation of media, this fact should be remembered.

In attempts to sterilize beef serum by heating to 56 C. for two hours on four successive days, I found that certain cocci remained living. On the other hand, these cocci grown in broth were promptly destroyed within two hours at 56 C. From a batch of milk which had been put through the Arnold sterilizer for twenty minutes on three successive days, we obtained a streptococcus mitis which had withstood the high temperature of flowing steam.

In order to further test this protective quality of serum, we used beef serum diluted one-half with water and sterilized by filtration. To this dilute serum we added a number of strains of streptococci, allowed the tubes to stand for ten days at the room temperature, and then heated the tubes to a temperature of 58 C. for twenty-three hours. Only one strain of streptococcus of four used survived the experiment. This strain had been recently isolated from the feces of a patient with dysentery and was of the streptococcus fecalis type.

A similar experiment, using ascites fluid and leaving the organisms in contact for only three days, showed that this same strain of streptococcus fecalis survived heating to 60 C. for six hours. No growths were obtained from any of the other thirteen streptococci used in this experiment. On a number of occasions, in attempts to isolate the bacillus Welchii from feces and other mixed materials by heating to 80 C. for 30 minutes, I have encountered in the cultures, streptococci, as well as the gas bacillus.

17. Handb. d. path. Mikroorg., 1912, 4, p. 453.

This work will be continued in an endeavor to determine the relationship between the relatively high resistance to heat of certain strains in albuminous fluids to the time of contact, the character of the fluid, the percentage of albumin, the number of organisms added, and other conditions that may have some bearing on the problem.

Ayers and Johnson,¹⁸ in a study of bacteria which survive pasteurization, have made some interesting observations on the resistance of bacteria in milk to high temperatures. These authors have shown that in heating milk to 62.8 C. there was little difference in the bacterial reduction produced by heating for one-half hour and by six hours' heating. The further study of their experiments, in heating samples of milk for a half hour at temperatures of 76.7, 82.2, 87.8, and 93.3 C., shows that their acid-forming group of bacteria, in a high percentage (80.91) survives the temperature of 76.7 C. The percentage is, however, greatly reduced at 82.2 C., and the higher temperatures. In a study of the cultures plated out from milk after pasteurization at 62.8 C. they noted that 89.39 per cent. of the acid-forming group of bacteria were cocci which produced no liquefaction of gelatin. The fermentation reactions of these and further cultures from two series, after heating to 60 C. and 65.6 C. for thirty minutes, would indicate that many of them were streptococci, although the authors do not state the morphologic arrangement of the cocci. This is all the more probable when we consider the frequency and high percentage of streptococci occurring in milk. By the typical lactic acid bacteria as shown in their table of reactions, the authors, no doubt, refer to the so-called streptococcus lacticus.

Frequent attempts have been made to separate the virulent streptococci from the non-virulent forms by the use of various materials, such as lecithin (Fromme,¹⁹ Machtle,²⁰ and others), but without success. Floyd and Wolbach,²¹ following the method of Churchman, have tested out the relative longevity of a series of streptococci in contact with gentian violet and conclude "that susceptibility to gentian violet may be a crude indication of the virulence of the streptococcus." Krumwiede and Pratt²² show that "the streptococcus-pneumococcus group differ from other gram-positive bacteria in their ability to grow in the presence of amounts of dye sufficient to inhibit the other species," and that the green streptococcus is not as sensitive as the hemolytic strain to the effect of certain dyes.

It is to be recognized that many of the virulent streptococci are more sensitive to a host of influences detrimental to the life of bacteria than are the more saprophytic types or the so-called non-virulent streptococci. This does not, however, hold in all cases and these methods of division of streptococci are being discarded for classification purposes.

LONGEVITY IN SYMBIOSIS

The wide distribution of streptococci and their constant association with other bacteria in the respiratory and intestinal tracts of man and animals in health and disease, as well as their presence in milk and

18. *Bull. Bur. Animal Indust.*, No. 161, 1913.

19. *Zentralbl. f. Gynäk.*, 1909, 33, p. 1217.

20. *Ibid.*, 1911, 35, p. 388.

21. *Jour. Med. Research*, 1914, 29, p. 493.

22. *Jour. Exper. Med.*, 1914, 19, p. 20.

other foodstuffs, make it of peculiar interest to learn something of their longevity in mixed cultures and in mixed infections. I have carried out a number of experiments with mixed cultures of different types of streptococci as well as with mixtures of streptococci and pneumococci and have found that they may live together for long periods of time if frequently transferred. Under unfavorable conditions, such as drying, etc., one or the other type disappears.

STREPTOCOCCI WITH STREPTOCOCCI

In my first experiment I used a strain of streptococcus pyogenes (570) from the pus of meningitis, and a strain of streptococcus fecalis (224) from an old empyema. These cultures were thoroughly mixed on a blood agar slant. Daily transfers were made to fresh blood agar slants and careful notes were kept each day of the appearance of the culture. Blood agar plates were made from time to time, colonies picked, and the freshly isolated cultures were tested for their fermentative reactions. These remained unaltered and distinctive. In this mixed culture hemolysis was the predominant feature up to the fifteenth transfer. On this transfer the blood, in general, was turned green, and with only two areas showing hemolysis. Further transfers showed the gradual return of the general picture of hemolysis, alterations were noted, a few green colonies appearing here and there on the slant at different intervals. On the twenty-ninth transfer there appeared numerous green colonies, but for the next ten transfers only hemolysis was to be seen. In plating on the surface of blood agar plates from these mixed cultures, two methods were used: (1) plates were smeared directly from the blood agar slants; and (2) plates were prepared after a preliminary growth in serum broth.

Several interesting observations were made. Direct plating from blood agar to blood agar usually showed a preponderance of hemolytic colonies and it was difficult at times to find isolated green colonies. Moreover, in picking from plates, showing both green and hemolytic colonies, the transfers from green colonies always proved to be pure, while transfers from the hemolytic colonies were sometimes mixed with the green strain. This is not to be wondered at, when we consider that the two organisms affect the blood in different degrees, one altering the hemoglobin and storing it in its colonies, the other bringing about hemolysis. The streptococcus with its more destructive action on red blood-cells will overshadow the character of the other type when the two are growing together. Moreover, the colonies of streptococcus fecalis are much smaller than those of the streptococcus pyogenes and were often observed within the hemolytic zone and in contact with the pyogenes colonies. The second method of plating, by growing in serum broth before smearing on the plates, allows the cocci to become well separated and the colonies developing are found to be pure. The

number of green colonies developing by this method is also greater, and their presence in the mixture is more easily determined.

STREPTOCOCCI WITH PNEUMOCOCCI

Parallel experiments were made with a strain of pneumococcus (556) obtained from a pneumonic lung at autopsy, and a strain of streptococcus fecalis (369) from feces of a patient with chronic constipation. This strain of streptococcus fecalis produced vegetations on the mitral valve of a rabbit after intravenous injection. The cultures were mixed as in the above experiment and daily transfers of the mixture were made. The pneumococcus colony is larger and more watery than that of the streptococcus fecalis, which is very small and dry. The results show that these two types grow together for long periods and can be reisolated in pure cultures. I encountered the same difficulty as previously noted in isolating colonies on the blood agar plates made directly from the blood agar slants. Transfers from the small green colonies proved to be pure, but those from the larger watery colonies quite frequently showed a mixture. Preliminary growth in serum broth eliminated this difficulty. The growth of the mixture gave the moist appearance of the pneumococcus culture up to the sixteenth transfer and from then until the thirty-ninth showed a more or less regular increase in the dry dotted characteristics of the streptococcus colonies, while here and there a more prominent colony was seen, supposedly of the pneumococcus. The colonies reisolated throughout the experiment were always tested for their fermentative reactions and never showed any alteration from the original strains.

The mixture, after being kept in the incubator for different lengths of time, was tested as a mixture in the carbohydrate serum broth media and usually gave the combined fermentation reactions of both strains. However, from some of the older mixed cultures only the reactions of the streptococcus fecalis were noted.

The pneumococcus remained living up to the last transfer, but it was often impossible to reisolate it from the older cultures. The ability to separate it from the mixture varied apparently with the condition of drying of the medium.

Eight dextrose serum broth tubes were inoculated from the fifth blood agar transfer of the mixture. These were grown for three days at 37 C. Blood agar plates were then made from the dextrose serum broth, but none showed any growth of the pneumococcus. Eight of the largest colonies were picked but they all proved to be the streptococcus fecalis. The high acid production in this medium, due to fermentation, would probably account for the suppression of the pneumococcus.

The growth from these eight tubes was separated by centrifugation, suspended in salt solution, divided equally, and injected into the peritoneal cavity of two guinea-pigs. After forty-eight hours, the guinea-pigs were killed, plates were made from the hearts' blood and 2 to 3 c.c. of blood injected directly into fresh guinea-pigs. The results are shown in Table 1. The pneumococcus was isolated in pure culture. A streptococcus mitis was isolated from the heart's blood of one animal. This result is discussed in a later part of this paper. All the colonies on the blood agar plates from the heart's blood were picked, and the cultures studied for their fermentative reactions. The streptococcus fecalis was not recovered.

The results of these experiments allow us to conclude that the relative longevity of streptococcus fecalis is greater on artificial media

than that of the pneumococcus, and that in the animal body the invasive power of the pneumococcus is greater than that of the streptococcus, or expressed in a different way, the streptococcus predominates on culture media while the pneumococcus gains the ascendancy in the animal body.

SYMBIOSIS WITH OTHER BACTERIA

The symbiotic relationships of the streptococci with other types of bacteria are also of great interest. In the intestinal and upper respiratory tract the streptococci are practically always present, living in closest contact with a host of other bacteria. Cultures from the peritoneal cavity following perforation of the intestines demonstrate that hemolytic streptococci are present in abundance, although cultures from the feces usually show that green-producing streptococci are in the greater numbers. With the bacillus diphtheriae, symbiosis is frequent and important. In obtaining pure cultures of the anaerobes and many other bacteria, streptococci are often difficult to eliminate. There are indeed a few organisms with which one or the other of the streptococci will not live (Gotschlich,²³ Rettger²⁴).

Working with a strain of subtilis bacillus, which contaminated mixed culture plates containing pneumococcus and streptococcus, I was able to show that the closest symbiotic relationship can exist for long periods. The colony of bacillus subtilis, which had developed on the more thickly seeded part of the plate, was transferred to a plain agar slant. After 24 hours there was a typical growth of an apparently pure culture of bacillus subtilis. A transfer was made to serum broth and after 24 hours' incubation, a blood agar plate was smeared with the growth. This plate showed the presence of streptococcus, pneumococcus and bacillus subtilis. The small colonies of the cocci were growing close to the subtilis growth, some touching the edge, others apparently within the subtilis colonies, while many were free, scattered in the vicinity. From all three types of colonies, transfers were made and cultures tested out on the various media. The pneumococcus and streptococcus were both recovered in pure culture. From some of the subtilis transfers streptococci were again isolated by plating through serum broth. After ten days in the incubator the mixture on the plain agar was again plated through serum broth and the streptococcus fecalis recovered. The pneumococcus had apparently disappeared. Seven transfers from plain agar to plain agar were made at various intervals and the streptococcus was isolated from each fresh culture of what appeared to be a pure growth of bacillus subtilis. The last plain agar transfer was made forty-one days after the contamination of the blood agar plate. From the second plain agar transfer, after it had been in the incubator for thirty-nine days, a blood agar plate was made through serum broth and the streptococcus was recovered. Colonies were picked from all plates and tested on the carbohydrate and other media. No alteration from the reactions of the original strains was noted.

A hemolytic streptococcus (*Streptococcus* XII), kindly sent by Dr. Rosenow, was used to test the effect of symbiosis with the bacillus subtilis. Four different strains of the bacillus subtilis group were used. Cross plates were made on blood agar. From the heavy growth of the bacilli, transfers were made to serum broths and from these, after incubation, blood agar plates were prepared. The hemolytic colonies on these plates were unaltered and were to be seen in closest contact with the bacillary colonies. From the serum broths plain agar slants were seeded and apparently pure cultures of the bacilli were demon-

23. Handb. d. path. Mikroorg., 1912, 1, p. 148.

24. Jour. Infect. Dis., 1905, 2, p. 562.

strated. After three days in the incubator, blood agar plates were again made through serum broth. The hemolytic colonies were unchanged on the third, while no growth of the streptococcus could be seen on the fourth. After nineteen days in the incubator on plain agar, blood agar plates from serum broth showed streptococci on only one of the plates. The hemolytic colonies on this plate were, however, typical. No green colonies were seen at any time in this series of experiments. The hemolytic colonies were often in closest contact with those of the bacilli.

From these experiments it would seem that the hemolytic strain of the streptococcus dies out more quickly than the green-producing type.

SECONDARY AND MIXED INFECTIONS

Symbiosis with other bacteria is a well-recognized method of raising the virulence of streptococci and pneumococci (Gotschlich). The study of mixed and secondary infections in human diseases demonstrates that the streptococci and pneumococci play an overwhelming rôle in these conditions. There are certain facts that must be borne in mind. The streptococci and the pneumococci enter the blood under many conditions of lowered resistance. The multiplicity of predisposing causes for pneumonia, the relative ease of streptococcal invasion in rheumatism, the frequency of secondary invasions in scarlet fever (Hektoen,²⁵ Klimenko,²⁶ Anthony²⁷), typhoid fever (Wassermann and Keyser²⁸), tuberculosis (Panichia,²⁹ Brown, Heise and Petroff³⁰), and other conditions of lowered resistance, the invasion in the dead body (Gwyn and Harris,³¹ Cannon,³² White³³) all bear out this statement. Moreover, it is not uncommon to find the pneumococcus as the cause of secondary conditions in streptococci infections, and vice versa.

SPONTANEOUS STREPTOCOCCUS INFECTIONS IN ANIMALS

What is true of man is also undoubtedly true for laboratory animals. Fatal epidemics caused by streptococci are not at all uncommon. A rapid review of the literature demonstrates this fact.

Charrin³⁴ found a streptococcus in a rabbit which had died from anthrax. Binaghi³⁵ isolated a capsulated streptococcus from a guinea-pig which died spontaneously. This was the first reported case of streptococcus mucosus infection. Boxmeyer³⁶ described a chronic infectious lymphadenitis of guinea-pigs which was caused by a streptococcus. Flexner³⁷ also found a streptococcus associated with a similar disease in guinea-pigs. Lamar³⁸ studied sixteen strains of this streptococcus which would indicate that the epidemic was a considerable one. Wittneben³⁹ studied an epizootic of guinea-pigs. Twenty-four died and a septicemia with pneumonia, pleuritis and pericarditis was found at autopsy. Streptococcus lanceolatus was isolated from these cases and was differentiated from the human strains of pneumococcus only by its easily stained capsule and its marked coagulation of milk. Weber⁴⁰ reports an epidemic of pneumonia

25. *Jour. Amer. Med. Assn.*, 1903, 41, p. 405.

26. *Centralbl. f. Bakteriöl.*, Abt. 1, Orig., 1912, 65, p. 45.

27. *Jour. Infect. Dis.*, 1909, 6, p. 332.

28. *Handb. d. path. Mikroorg.*, 1912, 1, p. 645.

29. *Baumgarten's Jahresbericht*, 1908, 24, v. 145.

30. *Trans. Nat. Assn. for Study and Prev. Tuberc.*, 1913, 9, p. 344.

31. *Jour. Infect. Dis.*, 1905, 2, p. 514.

32. *Die Bakteriologie des Blutes bei Infektionskrankheiten*, Jena, 1905.

33. *Jour. Exper. Med.*, 1899, 4, p. 425.

34. Quoted by LeGros, *Monographie des Streptocoques*, Paris, 1902.

35. Quoted by Shotmuller, *München. med. Wchnschr.*, 1903, 50, p. 849.

36. *Jour. Infect. Dis.*, 1907, 4, p. 657.

37. Quoted by Lamar, *Jour. Exper. Med.*, 1909, 11, p. 152.

38. *Jour. Exper. Med.*, 1909, 11, p. 152.

39. *Baumgarten's Jahresbericht*, 1907, 23, p. 149.

40. *Inaug. Dis.*, Munich, 1901.

among guinea-pigs. One-half of their rabbits died. At a later period the guinea-pigs became affected and thirty from a stock of fifty died. Streptococci were isolated from all thirty cases. Stephansky⁴¹ described a pneumococcus epidemic among guinea-pigs. The author isolated the pneumococcus from fourteen guinea-pigs, showing at autopsy a purulent and fibrinous inflammation of the lungs.

Selter,⁴² in his careful study of natural pneumococcus infection in laboratory animals, reported the isolation of pneumococcus lanceolatus from four guinea-pigs, one inoculated fourteen days before death with a pseudodiphtheria culture, another four days before, with killed tubercle bacilli, and a third after an inoculation two days before with a strain of bacillus dysenteriae. The fourth died spontaneously. Four rabbits also died with coccidiosis and pure cultures of pneumococcus were obtained from the cysts. The following winter another outbreak occurred in which one rabbit succumbed on the fourth day following inoculation with actinomyces, and five guinea-pigs died spontaneously or after the injection of cultures of different organisms. From these six animals pneumococci were also isolated. Lantz⁴³ speaks of sudden changes of temperature and improper ventilation as common causes of pneumonia in guinea-pigs. It is an extremely fatal disease in these animals. Salomon,⁴⁴ in his study of carbohydrate fermentation by the streptococci, includes in his list three strains of pneumococcus from an epidemic in guinea-pigs. Kutschera⁴⁵ found a streptococcus in an epidemic among white mice. Weil⁴⁶ in his investigations used, among other streptococci, a strain obtained from an epidemic in mice. Wherry⁴⁷ reports the finding of streptococci in two white rats. Andrewes and Horder⁴⁸ recovered from a mouse, inoculated with a pneumococcus, a gelatin-liquefying strain of streptococcus. These authors emphasize the liability of error in the use of animal passage for experiments on the constancy of the fermentation tests. Lamar⁴⁹ studied streptococci from three cases of fatal septicemia in monkeys, and pointed out the great importance of first understanding fully the disease of the laboratory animals.

In my experience, streptococci of various types have been obtained from guinea-pigs injected with other bacteria, and from guinea-pigs which died spontaneously. In Tables 1 and 2 the results of my experiments are given in condensed form. From these examples, as well as from the number of reports cited from the literature, it can reasonably be inferred that an invasion of the animal body by the streptococcus-pneumococcus group occurs under natural conditions, as well as from the injection of cultures of various bacteria. The strain of streptococcus mitis recovered from the heart's blood of Guinea-pig 6 was not, I believe, either a transformed pneumococcus or an altered streptococcus fecalis any more than the streptococci from the heart's blood of Guinea-pig 8, and those from the peritoneal cavity of Guinea-pigs 9 and 13 were derived from the dead or living cultures of the

41. Quoted by Selter, *Ztschr. f. Hyg. u. Infektionskrankh.*, 1906, 54, p. 347.

42. *Ibid.*

43. *Farmers' Bull. from U. S. Dept. Agricul.*, No. 525.

44. *Inaug. Dis.*, Kiel, 1901, 8.

45. *Baumgarten's Jahresbericht*, 1908, 24, p. 136.

46. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1911, 68, p. 346.

47. *Jour. Infect. Dis.*, 1908, 5, p. 515.

48. *Lancet*, 1906, 171, p. 708.

49. *Jour. Exper. Med.*, 1909, 11, p. 152.

TABLE 1
STREPTOCOCCI RECOVERED FROM INOCULATED GUINEA-PIGS

No.	Intraperitoneal Injection of	Result	Characteristics of Isolated Streptococci					
			Chains	Hemolysis	Lactose Serum Broth	Mannite Serum Broth	Salicin Serum Broth	Inulin Serum Broth
1	5 c.c. serum broth cultures of streptococcus from vagina†	Death after four days; streptococci isolated from peritoneum	Long	Green	+	+	+	+
2	5 c.c. serum broth culture from Guinea-pig 1	Death after 3 days; streptococci from peritoneum	Long	Green	+	+	+	+
3	Mixed culture of pneumococcus and streptococcus fecalis	Killed after 48 hours; cultures sterile
4	Same as No. 3	Killed after 48 hours; pneumococci from blood	—	Green	+	—	+	+
5	3 c.c. of blood from No. 4	Killed after 48 hours; pneumococci from blood	—	Green	+	—	+	+
6	3 c.c. of blood from No. 5	Killed after 48 hours; streptococci from blood	Medium	—	+	—	+	—
7	2 c.c. of blood from No. 5.	Killed after 48 hours; pneumococci from blood	—	Green	+	—	+	+
8	2 agar slants of colon bacilli	Died in 15 hours; colon bacilli and streptococci from blood	Short Long Short Medium	— — — —	— — — —	— — — —	+++++	—
9	3 agar slants of colon bacilli heated 60 degrees for 1 hour	Killed in 48 hours; streptococci from peritoneum
10	20 c.c. dextrose serum broth culture of streptococcus 659	Killed in 48 hours; cultures sterile
11	50 c.c. dextrose serum broth culture of streptococcus from guinea-pig intestine†	Killed in 48 hours; blood peritoneum; streptococci	Medium* Short**	—	+	+	+	—
12	Agar slant of colon bacillus	Killed in 16 hours; no growth
13	Peritoneal fluid from No. 12	Dead in 6½ hours; streptococci from peritoneum	Long	—	+	+	+	+

* From blood. ** From both blood and peritoneum.

† Hemolysis positive; lactose and salicin positive; mannite and inulin negative.

‡ Hemolysis negative; mannite and salicin positive; lactose and inulin negative.

TABLE 2
STREPTOCOCCI FROM GUINEA-PIGS DYING SPONTANEOUSLY*

No.	Anatomical Changes	Cultures	Characteristics of Isolated Streptococci					
			Chains	Hemolysis	Lactose Serum Broth	Mannite Serum Broth	Salicin Serum Broth	Inulin Serum Broth
1	Normal	Streptococci from cervical gland	Medium	+	—	—	+	—
2	Normal	No streptococci	—	—	—	—	—	—
3	Normal	Sterile	—	—	—	—	—	—
4	Pneumonia, pleuritis, congestion of small intestine	Streptococci (Peritoneum, Pleura, Blood)	Short† Long	—	+	+	+	—
5	Pneumonia	Streptococci from pleura and blood	Long	+	+	—	+	—
6	Pneumonia	Streptococci from pleura and blood	Long	+	+	—	+	—
7	Pneumonia, pleuritis	Streptococci from pleura and blood	Long	+	+	—	+	—
8	Pregnancy	Pseudodiphtheria bacilli from blood	..	+	+	+	+	..

* These guinea-pigs were those dying spontaneously from a stock of about 700.

† From peritoneum.

‡ From pleura and blood.

TABLE 3
CHARACTERISTICS OF STREPTOCOCCI OBTAINED FROM GUINEA-PIGS.

Source	Chains	Hemolysis	Lactose Serum Broth	Mannite Serum Broth	Salicin Serum Broth	Inulin Serum Broth	Notes
Intestinal Contents	Medium	—	—	+	+	—	See Nos. 1 and 2, Table 1.
Intestinal Contents	Medium	—	+	+	+	+	See Nos. 9, 11, 13, Table 1, and No. 4, Table 2.
Intestinal Contents	Medium	—	+	+	+	—	See No. 8, Table 1.
Throat	Short	—	—	—	+	+	
Throat	Short	—	+	+	+	+	
Throat	Long	—	+	+	+	+	

bacillus coli. In 1910, I recovered from the peritoneal cavity of a guinea-pig injected with a hemolytic streptococcus from the vagina, a non-hemolytic streptococcus which fermented inulin. I concluded that this organism had come from the animal.

Members of the streptococcus-pneumococcus group are found regularly in the intestinal and upper respiratory tracts of most animals (Selter,⁵⁰ De Gasperi,⁵¹ and others). I have isolated members of this group from the intestines and upper respiratory tract of a number of normal guinea-pigs. The cultural characteristics of these streptococci are shown in Table 3.

Many investigators have demonstrated by experiments the comparative ease with which organisms pass from the intestinal canal of animals into the bloodstream and the organs of the body. Loiseleur,⁵² Sacquepee and Loiseleur,⁵³ Basset and Carre,⁵⁴ Garnier and Simon⁵⁵ and others have shown that various methods of lowering the resistance by heat, cold, inanition, overexertion, injections of toxins, hypertonic solutions, dilute acids, and other conditions, lead to a bacteremia.

Adami⁵⁶ has shown that the tissues are potentially, but not actually, sterile. In animals fed with various cultures of bacteria these can be obtained, after a few hours, from the various organs of the body, for during the digestive process bacteria are absorbed along with the food, especially when much fat is present. Acute congestion of the mucosa of the intestines, however brought about, favors the passage of bacteria.

Simmonds⁵⁷ found that, after injecting rabbits with 500,000 to 2,500,000 killed streptococci, he had first a fall, then a rise in the opsonic index, and it is well known that injections of large numbers of bacteria regularly bring about a fall in the opsonic index. Young animals have been shown to be more susceptible to invasion than the full grown.

MUTATIONS

Buerger and Ryttenberg⁵⁸ believed that they had observed newly acquired properties in many of their strains of pneumococcus. It is interesting to note that in their first case the blood culture tested was taken two days before death and that this culture was readily converted from an atypical into a typical pneumococcus by passage through mice. A strain isolated from a metastatic abscess of this case showed the same general cultural characters. This latter strain could not be converted, and the former, after the lapse of one month, was also non-convertible. The authors state that the colonies from this blood culture showed "some ring forms" and that the morphology was "like degenerated pneumococci." I would conclude that the writers were dealing with a mixed infection in the blood culture, and a pure infection in the metastatic abscess. This explanation, based on the results of my experiments, as recorded in the earlier part of this paper, as well as on experience with mixed infections with pneumococcus, will serve to clear up the apparent confusion of the other cases cited by these authors.

Rosenow⁵⁹ in a recent communication claims that he has been able to transmute the pneumococcus to streptococci of various types, and vice versa.

50. *Ztschr. f. Hyg. u. Infektionskrankh.*, 1906, 54, p. 347.

51. *Centralbl. f. Bakteriöl.*, 1, Orig., 1911, 57, p. 519.

52. *Baumgarten's Jahresbericht*, 1906, 22, p. 812.

53. *Ibid.*, 1907, 23, p. 703.

54. *Ibid.*, 1908, 24, p. 962.

55. *Ibid.*, 1909, 25, p. 969.

56. *Jour. Amer. Med. Assn.*, 1899, 32, p. 1509; *Br. Med. Jour.*, 1914, 1, p. 177.

57. *Jour. Infect. Dis.*, 1907, 4, p. 595.

58. *Ibid.*, p. 609.

59. *Ibid.*, 1914, 14, p. 1.

Schereschewsky⁶⁰ believes that the streptococcus pyogenes is closely related to the pneumococcus. He was able to bring about very marked morphologic changes and believed he was able to change one form into the other. Schereschewsky's results have not been accepted as indicating any fundamental alteration of these organisms.

Rosenow has gone much further and he is convinced that "the transformations of some of the strains are complete by every known test." He has not only altered the morphology, the formation of capsules, and the fermentative powers, but also the specific immunity response, and the more or less specific pathogenic powers. The results of my experience and those of numerous other workers would tend to throw doubt on many of his interpretations.

In the detailed description of some of his experiments I am able to follow some of the steps of his transmutations. In Strain 595 he had a hemolyzing culture which, after growing for over a year and showing no important change during this time, was transferred to blood agar slants and left in the incubator twenty-nine days until "the media were very dry." Transfers were now made to fresh blood agar, and after eight days at 37 C. the surface of a smeared blood agar plate showed two types of colonies, one hemolyzing, and the other not. This would appear to the ordinary observer to have been either from a survival of a non-hemolyzing strain, as shown in my experiments, or to have been a contamination. These non-hemolyzing colonies were first noted on November 15. On November 17, four colonies of each variety were plated on blood agar plates. One of the non-hemolyzing colonies, thus plated, showed on November 19 two types of colonies, adherent and non-adherent. On this same date, before these two types of colonies had been studied further, Rosenow injected four small rabbits (600-750 gm.) with the growth from 20 to 60 c.c. of ascites dextrose broth culture of this streptococcus viridans strain. One colony from the blood of one of these rabbits was used for the further transmutation into pneumococcus. On November 21, transfers were made from the culture of an adherent green colony to blood agar and Loeffler's serum. After seventy-seven days the growth on Loeffler's serum yielded both hemolyzing and non-hemolyzing colonies. Is this evidence of reversion or the indication of a mixed culture? The author does not give the fermentative and other characteristics of these various strains when first isolated as a streptococcus viridans, when recovered from the first injected rabbit, nor after the first two guinea-pig injections. The transformed pneumococcus, after being subcultured for five months, yielded a hemolytic strain which showed fermentation of mannite, a reaction not shown by either the original hemolytic strain or the pneumococcus. In such an important question as the conversion of such well differentiated organisms as the pneumococcus and the hemolytic streptococci, full and complete cultural and other characters should be given at every step in the alteration. That the organisms at the beginning and at the end of the experiment are quite different in every important character we have no doubt, but if transmutation has really occurred, we must know when the changes took place.

The transmutations by growth with the bacillus subtilis we have been unable to confirm, although we used a hemolytic strain (XII) kindly sent me by Dr. Rosenow, and cultured it in intimate contact with four strains of the subtilis bacillus. We were, however, able to show that a strain of streptococcus fecalis grew with the bacillus subtilis for long periods of time, and could be best recovered by plating on blood agar after first growing the mixture in serum broth. Strain XII as a hemolyzer, had been converted by Rosenow into strep-

60. *Centralbl. f. Bakteriol.*, Abt. 1, Orig., 1909, 49, p. 72.

Streptococcus viridans by growth in symbiosis with the *Bacillus subtilis*. The full characteristics of the three varieties of colonies, obtained from this strain after growth on ascites-dextrose agar, are not given. It is to be noted that ascites fluid seemed to be necessary for the alteration into the rheumatic types of streptococci with this and other strains discussed under "the effect of growth in a high oxygen pressure." The author used in his media, in the study of the etiology of acute rheumatism, ascites fluid which yielded uniform and frequent positive results. The very uniformity is striking. In one case he obtained two colonies per 20 c.c. from the right knee, two colonies per 12 c.c. from the left knee, three colonies per 2 c.c. from the wrist, and ten colonies per 0.05 c.c. of bloody fluid from a red, tender, swollen area over the lower end of the ulna. Is it possible that the ascites fluid was not sterile? The method of heating to 60 C. for twenty-four hours to obtain sterile fluid is not as certain as careful filtration. In testing such a fluid for the presence of a few organisms, large quantities must be employed. Recently I obtained from a peritoneal swab, taken at operation, a strain of streptococcus which showed a marked tendency to anaerobism. It grew best in the depths of carbohydrate serum agar media and did not develop on the surface till after several transfers. It did grow, however, in very minute colonies on fresh blood agar slants. Unfortunately, the resistance to heat of this streptococcus was not tested. Such an organism would be readily overlooked in testing the sterility of human fluid for use in culture media.

Strain 734 was claimed to have been changed by aerobic culture in distilled water from a non-mannite green-producer into a hemolyzer and mannite fermenter. Its virulence was also distinctly greater than before. The raising of virulence by growth in water is contrary to the experience of all workers in water bacteriology.

From my experiments with guinea-pigs, and the results of the other investigators cited, I would be unwilling to believe that the recovery of green streptococci from a joint, or the pericardium, or other cavities of animals injected with large doses of a hemolytic streptococcus, is sufficient evidence to conclude that the recovered organism is the same as that injected.

The altered form of Strain 734, which was subsequently changed to pneumococcus, was derived from the pericardium of a rabbit. Strain 736 "in the joint of one rabbit, in its second passage, lost the power to hemolyze and produced green colonies instead." When Strain B, a "pure line," which had acquired the power to hemolyze blood agar, was recovered from the joint of one rabbit it was found mixed with green streptococci. This latter strain was then transformed into pneumococcus. Strain R 51A was originally a pneumococcus, but had acquired the power on dried blood agar to hemolyze blood for the first time in over ten years. This strain was passed through sixteen rabbits. The joint culture from the sixteenth animal showed both green and hemolyzing colonies. A hemolytic streptococcus, derived from a pneumococcus, was injected into a rabbit, which five days later was found dead. Joint fluids yielded three green and no hemolyzing colonies.

The number of guinea-pigs used, before the transmutation of strains of streptococcus viridans into pneumococcus took place, varied greatly. My interpretation of this fact is that the invasion from the animal varied with the different animals, or the invading organisms were not recovered until they had gained a numerical ascendancy.

Andrewes,⁶¹ in an excellent paper, has discussed the nature and degree of specific differences amongst bacteria, and his points are well

61. *Lancet*, 1906, 2, p. 1415.

worth consideration for maintaining a conservative view of the questions of differentiation of bacteria.

CONCLUSIONS

Streptococci vary within wide limits in their longevity on artificial media. They also show wide differences in their sensitiveness to the effects of desiccation.

The presence of acid, produced in media containing carbohydrates, is very detrimental to the life of streptococci. The widest variation is exhibited among different strains to this effect of acid.

Many strains of streptococci are resistant to high temperatures over prolonged periods of time, more especially when protected by albuminous fluid as in milk, beef serum and ascites fluid.

Streptococci of all forms live in closest symbiotic relationship with many other bacteria.

Hemolytic streptococci, growing on blood agar in mixed culture with a green-producing form, show an apparent predominance, and pneumococcus and streptococcus, in mixed cultures, show that the growth of the pneumococcus may be overshadowed by that of the streptococcus.

Plating on blood-agar after a preliminary growth in serum broth is a more certain method for obtaining pure cultures than direct plating from blood-agar to blood-agar.

The injection of known mixed cultures of pneumococcus and streptococcus, which culturally appear to be pure streptococcus, shows that the more invasive form, the pneumococcus, survives.

In mixed cultures of pneumococcus and streptococcus fecalis the former disappears first, as the result of drying of the media.

Strains of green streptococci will live for long periods in mixed cultures with the bacillus subtilis, while the pneumococcus and hemolytic streptococci die out more quickly.

The growing of hemolytic streptococci with cultures of the bacillus subtilis for long periods has no effect in altering its hemolytic properties.

Streptococci and pneumococci invade the body under many and various predisposing conditions. They are also by far the most frequent secondary invaders.

These organisms spontaneously invade the bodies of our laboratory animals bringing about fatal epidemics. Various predisposing causes make these invasions possible.

The intestinal and upper respiratory tracts of guinea-pigs contain different forms of streptococci.

Injections of bacteria into guinea-pigs, especially young animals, bring about an invasion into the body cavities and the blood-stream of organisms, more particularly of various strains of streptococci derived from the normally infected regions of the animal.

Isolation from animals of streptococci, differing in important characteristics from those injected, cannot be taken as proof of a change in the characteristics of the injected streptococcus within the animal body, owing to the ease with which streptococci are able to enter the tissues from the intestines or respiratory tract of animals as well as man.

In attempts at transmutation all the characteristics of the organisms must be given at every stage of the experiment.

OBSERVATIONS ON THE GROWTH OF THE GONOCOCCUS AND THE STAPHYLOCOCCUS ALBUS FROM THE URETHRA IN PLATE CULTURE (A CRITICISM OF WARDEN'S WORK)*

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The identity of some well known bacteria is being doubted by progressive bacteriologists. Variations in the so-called "characteristic properties," which serve as criteria in the differentiation of bacteria, suggest the possibility that our predecessors have confused bacteriologic terminology by assigning names to many variations of a few bacteria, when growing in widely dissimilar environment. Much valuable knowledge will undoubtedly be obtained by a review of the subject of bacteriology in the new light. Yet this must be done with care. The Gram stain, a variable quantity in itself, and the elusive phantom, morphology, cannot give conclusive evidence that two bacteria, heretofore considered different, are the same, as has been contended in Warden's publications of last year, which we are about to discuss.

Warden,¹ in his studies of the gonococcus and the staphylococcus albus from the urethra, concludes that "Many, if not all, of the gram-negative, intracellular, biscuit- or coffee-bean shaped cocci, observed in the purulent discharge in acute gonorrhea, which are regarded as gonococci, and which serve as criteria of diagnosis, are not gonococci, but belong to the staphylococcus group. The provisional name of *Staphylococcus urethrae* is suggested for this coccus."

As a basis for this conclusion, Warden cites his observations on the Gram staining properties of the staphylococcus albus, obtained from urethral pus: (1) when grown on the usual media in pure culture and in mixed culture with the gonococcus; (2) when within leukocytes, in phagocytosis experiments made to obtain intracellular organisms, and (3) when in colonies.

Pure cultures of the staphylococcus albus and gonococcus were grown on plate culture media spread in thin films on microscopic slides which permitted staining and study of colonies *in situ*.

* Received for publication, May 28, 1914.
1. *Jour. Infect. Dis.*, 1913, 13, p. 124.

Further peculiarities in Gram staining, together with changes in morphology of the same staphylococcus were observed when the organisms were in suspension in body fluids such as blood-serum, hydrocele fluid and ascites fluid; and in ferments, such as dilute pancreatin, leukocytic extract and gonococcus autolysate. These fluids are of significance in the study because the product of dead leukocytes or leukocytic extract, and the by-product of gonococcus growth, gonococcus autolysate, are present within the urethra in urethritis.

Briefly, Warden's observations are as follows:

1. In suspensions of staphylococci, the Gram staining properties of the cocci vary according to the nature of the fluid from which the suspension is made. Coverglass preparations made from this coccus in normal salt solution show the familiar gram-positive staphylococci, even in suspensions which have stood at room temperature for weeks. But old suspensions three to six hours old in serum, hydrocele fluid, ascites fluid, weak pancreatin solution and gonococcus autolysate show gram-negative forms of the still viable cocci.

2. The intracellular diplococci in gonorrheal pus are probably gram-negative staphylococci in diplococcal form. Phagocytosis experiments demonstrate both the ease with which staphylococci, especially when in the gram-negative state, are taken up by leukocytes, and the difficulty experienced in ingesting gonococci. Even though the same conditions exist in the urethra as in the laboratory, the gonococcus is not an organism to be expected within the cells in gonorrheal pus.

3. The staphylococcus grows for the first few hours as a gram-negative organism. When staphylococci are planted on plate slides and stained at various stages of incubation, small, young colonies, up to 8 to 12 hours of age, all show gram-negative cocci. Older colonies show gram-negative bases, where the cocci are still in contact with the culture media, but in those portions of the colony which are elevated above the surface of the media, the cocci are gram-positive.

Before proceeding to a discussion of these findings, I wish to mention some general considerations pertinent to the cultivation of the gonococcus and the staphylococcus albus, some of which have been discussed in Warden's articles.

Staphylococcus albus and gonococcus colonies, when grown on the usual media, are unmistakably different. Microscopically, the large, slightly elevated, whitish colonies, which are seen at the end of eighteen

to twenty-four hours incubation, are in no way to be confused with the smaller, transparent, dew-drop colonies of the gonococcus. Colonies of gonococci more than twenty-four hours old become more opaque, but never white to such an extent that one would confuse the growth with that of the staphylococcus on the same media. Microscopically, the two types of colonies are distinguishable. These small colonies are best compared when the mixed suspensions of gonococcus and staphylococcus are planted on slide-plate media, such as Warden uses, and stained *in situ*. Eight- to twelve-hour growths are best for the purpose, since the colonies at this time are composed of but from one to three layers of bacteria. The staphylococcus colony appears as an irregularly bunched, elevated colony of cocci and diplococci, from two to three layers thick at the center to a single layer at the periphery, with coccal forms predominating. The gonococcus colony is smaller, flat and composed chiefly of diplococci arranged mosaically over the surface of the media in a single layer. Both types of colonies are gram-negative at this stage of incubation, but in spite of the similarity, are easily distinguishable by the arrangement of the cocci composing them.

Pure cultures of the gonococcus are seldom obtained from pus in gonorrheal urethritis. Pus from early cases of urethritis, from two to six days after the appearance of the discharge, usually shows a preponderance of gonococcus colonies and a much smaller number of staphylococcus colonies. In the late cases, two or more weeks from the appearance of the discharge, the growth is chiefly staphylococcus with a few gonococcus colonies.

Diplococcal forms are common in any rapidly growing coccus. The phenomenon is due to rapid growth with incomplete division at the time of observation. Perhaps in the best coverglass preparations, morphology may be sufficient for differentiation of these cocci from gonococci, but this is not the case where the bacteria are stained on plate culture media, as in Warden's experiments, or clinically, where other bacteria and much cell detritus is present.

The Gram method of staining bacteria is not absolutely reliable, and numerous variations in the reaction of the same bacteria in changed environment have been recorded. Eisenberg² has shown that gram-positive bacteria undergoing spontaneous degeneration, a process of bacteriolysis, become gram-negative. Also gram-positiveness of

bacteria is influenced by serum without the picture of bacteriolysis being presented. The cocci are still viable. The causal agent is not mentioned.

Schmidt,³ working with colon bacilli found the organisms gram-positive when occurring in fat stools. Cedarkreutz⁴ found some cocci and colon bacilli gram-positive in butter. Nikitin⁵ found that by treating some varieties of bacteria with fat-splitting ferments they became gram-positive. Grimme⁶ records the observation that weak alkalies, 5 percent hydrochloric acid, pepsin and trypsin will produce gram-negative bacteria.

Eisenberg⁷ expresses the opinion, from observation of the work of others as well as from his own study, that introduction of albuminous material into bacterial preparations and culture media produces gram-negative bacteria in some cases.

Of these observations, that which most concerns the question in hand is the effect of albuminous material on the Gram stain. Inasmuch as the gonococcus is difficult to grow in culture, except on special media containing blood-serum or its equivalent, albumin is present in Warden's experiments wherever gram-negative staphylococci are seen. The same condition results in his suspensions of the staphylococcus in body fluids, but is absent in the normal salt suspensions in which the cocci remain gram-positive. Similarly, gonococcus autolysate contains albumin as a requisite to the growth of the gonococcus which produces it. Experimental evidence has already been indicated to show the possibility of ferments being further factors in producing gram-negative bacteria in this case.

At the suggestion of Dr. H. Cabot and under the supervision of Dr. J. H. Wright, I have endeavored to repeat Warden's experiments exactly as described by him with the exception of two minor variations from his technic, viz.: (1) hydrocele agar was substituted for ascitic agar, since our cultures grew better on this media, as we prepare it in this laboratory, than on our ascites fluid agar; (2) the substitution of Bismarck brown for 1 percent carbol fuchsin as a counterstain.

3. Cited by Eisenberg.⁷

4. *Arch. f. Dermat. u. Syph.*, 1908, 93, p. 355.

5. *Arch. Russ. de Pathol.*, 1908, pp. 132, 162.

6. *Centralbl. f. Bakteriol.*, Abt. 1, O., 1902, 32, p. 1.

7. *Centralbl. f. Bakteriol.*, Abt. 1, O., 1910, 56, p. 193.

Our findings are essentially the same as those reported by Warden. gram-negative staphylococci are readily produced by treatment of gram-positive organisms with serum or body fluids, and by the ferment action of leukocytic extract, gonococcus autolysate, and pepsin. By phagocytosis of these gram-negative staphylococci, leukocytes containing organisms similar to those found in gonorrheal pus were produced. But gram-negative staphylococci in cells were not sufficiently characteristic to deceive the house officers of this hospital, and the diagnosis of gonorrhea was not once made. Gonococci, also, can be taken up by leukocytes, *in vitro*, but less readily than gram-negative staphylococci.

On plate cultures the staphylococcus grows in gram-negative form in cultures up to eight hours; then the cocci become gram-positive in all portions of the colony where the individual organisms are not in contact with the surface of the media, i. e., in that part of the colony which is elevated above the surface of the media.

Further experimentation with plate cultures revealed significant facts. A suspension of the same staphylococcus was made in normal salt solution. Coverglass preparations from this suspension showed all gram-positive cocci. With material from this suspension, plates were prepared and stained immediately without incubation. The cocci on the plate were gram-negative. If, however, the bacteria from another slide, like the first, be washed off into salt solution again, they stain gram-positive as before. Gram-negativeness appears to depend entirely on the presence of the staphylococci on the plate media. *Staphylococcus albus* and *aureus* from abscess, and streptococci obtained from the throat, were also found to be gram-negative on plate media, but gram-positive in water or salt solution suspensions.

If a small colony from a six- to eight-hour slide plate culture of *staphylococcus albus* is removed from its bed and washed in normal salt solution and the plate stained as usual by Gram's method, and coverglass preparations, made from the suspension containing the colony, removed, the colonies remaining on the plate are all gram-negative and the cocci in suspension gram-positive. These facts lead one to doubt that the staphylococcus normally grows gram-negative for a time, but to believe rather that the appearance of gram-negativeness is due to an artefact. If so, what is the causal factor? Extracts of hydrocele agar, which contain phosphates and other salts, do not produce gram-negativeness. Thin films of celloidin, when replacing

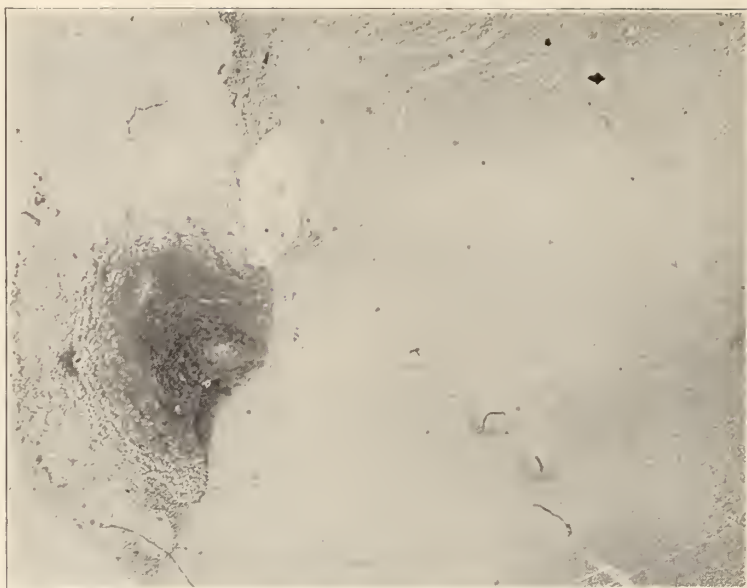


Fig. 1.—Low magnification (x12) of a field of *Staphylococcus albus* with the right half of the field coated with egg albumin. Cocci to the left of the albumin line are gram-positive, those to the right gram-negative. Field shown in Figure 2 indicated at X.



Fig. 2.—Edge of albumin field. Cocci to the left are not covered by albumin and stain gram-positive. The right half of the field shows gram-negative cocci beneath the albumin. x1,500.

agar on plate slides, cause no loss of gram-staining ability. Variations in the time allotted to each step in the performance of the stain makes no essential difference in the results, except that in some cases where methyl violet has been allowed to remain in contact with the bacteria for a considerable time, decolorization is difficult and the bacteria show dark centers with gram-negative peripheries.

While working with suspensions of staphylococci in hydrocele fluid which contained small flakes of albuminous material, I observed that in coverglass preparations all the cocci lying free in the field were

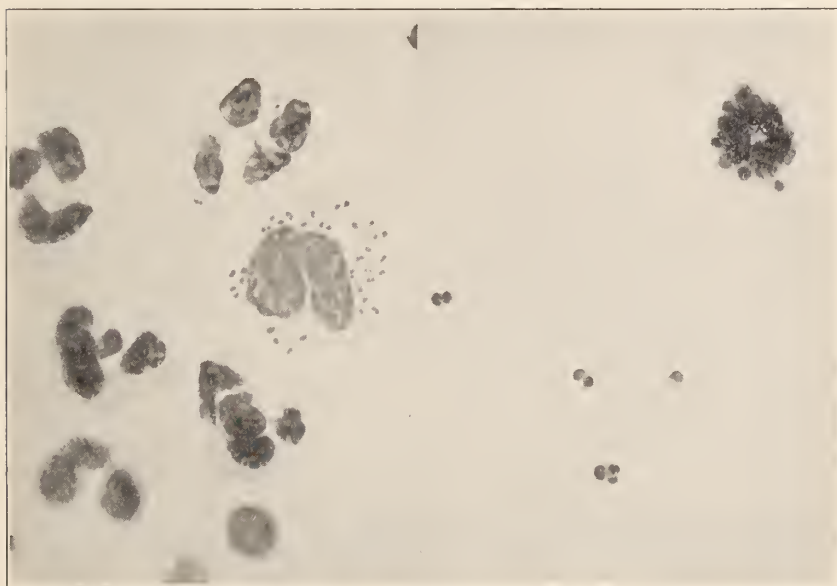


Fig. 3.—Left half of field shows intracellular gonococci from urethral pus. Right half of the field shows phagocytized gram-negative staphylococci. $\times 1,500$.

gram-positive, while those beneath the flakes were gram-negative. Also, in suspensions made in blood-serum which contained a few red cells in clumps, the cocci which were entangled in cell clumps and fibrin were gram-negative, but elsewhere gram-positive. These observations were made in newly prepared suspension where no incubation had taken place, nor had the preparations been allowed to stand for some hours — two conditions which Warden considers essential for the production of gram-negative staphylococci in serum. Albumin clearly interferes with the staining properties of these organisms. Experi-

mental evidence tends to show that by long contact with serum, the bacteria become sufficiently coated with albumin to respond abnormally to the Gram stain. That there is no permanent change in the bacteria themselves is demonstrated by the fact that after washing in salt solution they again become gram-positive. Gram-negative cocci are viable since cultures from suspensions in which gram-negative forms predominate show abundant growth.

Mechanical interference by albumin clearly explains the growth of gram-negative colonies from gram-positive cocci. A thin layer of albumin, derived from the media, covers the surface of the media to a sufficient depth to submerge the small colonies, but apparently does not in any way interfere with growth. When the colony attains sufficient size to rise above the high-water mark of albumin, the top stains gram-positive while the portion remaining below the albumin continues to stain gram-negative.

Gram-negative staphylococci can be prepared at will. If a dilute solution of egg albumin is applied to a portion of the surface of a coverglass preparation of staphylococci with a camel's-hair brush, and the Gram stain applied, it is found that in the portion of the preparation covered by albumin the cocci are gram-negative, elsewhere they are gram-positive.

Even though we grant Warden's hypothesis that conditions found within the urethra in gonorrheal urethritis, and laboratory conditions are essentially the same, we believe that he produces insufficient evidence for the sweeping conclusion that, "Many, if not all, the intracellular, gram-negative, biscuit- or coffee-bean shaped diplococci, commonly called gonococci," are staphylococci masquerading in gram-negative form.

He believes that gram-negative diplococcal forms of staphylococci are indistinguishable from gonococci when seen in the ordinary coverglass preparations from pus.

By phagocytosis experiments he shows that the gram-negative staphylococcus is more likely to be the organism within the pus cell than the gonococcus. As a further argument he states that the staphylococcus normally has a gram-negative stage of growth eight to twelve hours long, which stage is the one seen in acute gonorrhea. The chronic gonorrheas represent late stage gram-positive growth of the same organism.

He has failed to note the fact that the Gram method of staining bacteria does not give uniform results when applied to bacteria in unusual surroundings, especially in the presence of fats and albumin. The Gram stain, although supported by the doubtful aid of morphology, does not warrant the reliance placed on it.

He has also entirely neglected cultural evidence of the presence of the gonococcus in gonorrhea. The large proportion of gonococcus colonies and the few staphylococcus colonies seen in cultures taken directly from urethral pus in early cases of urethritis, seems to indicate beyond doubt that the intracellular diplococci "commonly called gonococci" and which produce gonococcus colonies, are not staphylococci; and that the reason so few staphylococcus colonies appear is that few are present in the pus. If the intracellular organisms are viable gram-negative staphylococci, which laboratory experiments show to be possible, there should be a growth of almost pure staphylococcus instead of gonococcus colonies. We have absolutely no evidence to show that staphylococci in their gram-negative state ever produce the gonococcus type of colony, or organisms which behave in culture like the gonococcus. The staphylococci present in acute urethritis pus may be in gram-negative form; indeed, there is reason to believe that they are gram-negative in pus with the high albumin content of acute urethritis pus.

I have shown that the staphylococcus does not have a short gram-negative stage of growth, but that this is an illusion produced by mechanical interference of albumin with the staining reaction of the bacteria, either by preventing penetration of the basic stain or interference with fixation of the stain. Plate culture media, which contains considerable albumin in added serum, hydrocele or ascitic fluid necessary to the growth of gonococci, and used by Warden for the staphylococcus also, is covered over with a thin layer of albumin deep enough to cover the small colonies completely and the bases of the larger ones producing a gram-negative reaction in these portions.

The morphology of cocci and diplococci, except perhaps in cover-glass preparations from pure culture, gives little aid in differentiation and is an even less valuable aid in pus with cell detritus. I have been unable to produce "pus cells" containing gram-negative diplococcal staphylococci which are indistinguishable from gonorrheal pus, although the resemblance was close in some cases.

SUMMARY

Gram-negative diplococcal staphylococci may be produced in the laboratory, and it is conceivable that they also occur in the urethra, but gram-negativeness is due to mechanical interference of albumin with the Gram stain.

The staphylococcus albus has no stage of growth in which it is a gram-negative organism. The appearance is due to an artefact.

The evidence presented by Warden that gonorrheal urethritis is due to the staphylococcus albus is based on erroneous interpretations of his observations and is fallacious.

SOME STRUCTURAL TRANSFORMATIONS OF THE BLOOD-CELLS OF VERTEBRATES *

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PLATE 2

The current descriptions of the structure of vertebrate blood-cells are largely based on material which has been prepared by the smear method and coagulated by heat, alcohol, or mixtures of electrolytes. The activities of white cells, that are generally recognized, are slow ameboid movement by means of either blunt or fine pseudopods, and the engulfment of objects by means of active pseudopods.

The numerous recent investigations on blood-cells made with the aid of the dark-field method of illumination have added interesting points of detail to previous knowledge of the form and activities of the cellular elements of the blood. No convincing evidence has been brought forward to show that the widely held notions about vertebrate blood-cells are seriously at fault. This fact is clearly due to the confusion caused by the presence of fibrin granules and threads in preparations made by the usual methods. In fact, many of the recent investigations are nothing more than the description of attempts to interpret the complex and variable images of the structural elements of blood clots which are produced by oblique illumination on a dark background.

By the employment of a special mounting or culture medium, I have been enabled to determine that both white cells and erythrocytes of all classes of vertebrates may undergo many marked structural transformations, which are of a reversible nature. In this paper, the more important of these transformations, so far observed, will be described.

MATERIAL AND METHODS

The blood-cells of fishes, amphibians, reptiles, birds, and mammals have been studied. Particular attention has been given to the blood-cells of the frog, the rabbit, and man. The actual species, so far used, include common mammals, as the mouse, goat, guinea-pig, rabbit, monkey (*Macacus rhesus*), and man, and in addition the goldfish

* Received for publication June 1, 1914.

(*Carassius auratus*), bullfrog tadpole (*Rana catesbiana*), leopard frog (*Rana pipiens*), painted turtle (*Chrysemis picta*), and pigeon (*Columba livia*).

A detailed description of the microdissection method employed will soon be published. It will suffice to state that the method has been developed to the point where it is adequate for the determination of the colloidal structure of living matter. Such structural elements of living cells as nuclei, nucleoli, pieces of chromatin networks, globules, granules, chromosomes, and spindle fibers may be cut out of living animal and plant cells and their exact physical properties and relationships determined. A new microdissection apparatus has been designed embodying the well-known ball-bearing principle. The three micrometer screws in this apparatus are made with the accuracy common to the best microtomes. Special achromatic and apochromatic condensers, giving long working distances, are essential for the dissection of cells under oil immersion objectives.

A 2 mm. Zeiss objective of 1.40 N. A., Comp. Ocs. 6 and 8, and W. Watson and Sons' holoscopic oil-immersion condenser, have been used for observing living blood-cells and for microdissections. The dark-field method of illumination has also been employed with notable success.

Liquid plasma, diluted with Ringer's fluid, which contains sufficient hirudin to permanently prevent coagulation, has been generally employed as the mounting medium. The technic which I have found best for the preparation of the blood-cells for observation and microdissection is very simple. About one milligram of hirudin is added to 2 or 3 c.c. of sterile Ringer's fluid in a small flask. About 0.5 c.c. of this mixture is drawn into a pipet which is applied to a drop of fresh blood obtained by the usual puncture method. The blood and Ringer's fluid are quickly blown into the flask containing the Ringer's fluid and hirudin. This operation is repeated until the Ringer's fluid has a definite red color. If sufficient hirudin has been added to the Ringer's fluid, and reasonable deftness be shown in getting the blood well mixed with the Ringer's fluid, neither fibrin granules nor threads are formed and the platelets are not clumped. Microscopical examination of a drop of this mixture should show the blood-cells evenly distributed through the liquid, and widely enough spaced to give optimum conditions for the exhibition of activities by the cells.

This use of hirudin is based on the work of Bodong,¹ and Abel, Rowntree, and Turner.² Bodong has demonstrated the harmlessness of even large quantities of hirudin for rabbits, and Abel, Rowntree, and Turner could show no detrimental action of this substance on dogs. Hirudin has given equally satisfactory results with the blood-cells of all vertebrates so far tried. The simple method of using hirudin that I have described is so satisfactory that it may find wide usage among those interested in the microscopic examination of fresh blood. Further, it seems that plasma, kept liquid by the employment of hirudin, used either undiluted or diluted with an appropriate salt solution may prove of great value in the *in vitro* cultivation of many other types of cells, besides the formed elements of the blood.

The results obtained by the hirudin method have been controlled by the use of defibrinated blood and by the addition of citrate and oxalate to prevent coagulation. While similar physical changes of the blood-cells may be observed when these more usual methods are employed, no evidence could be adduced to show that such methods of handling blood-cells for *in-vitro* studies have anything but disadvantages as compared with liquid-plasma and Ringer's fluid containing a trace of hirudin.

The observations and microdissections of the blood-cells of cold-blooded vertebrates were made at room temperature; those of birds and mammals at both room temperature and at body temperature. The structural transformations of blood-cells are so similar for all classes of vertebrates that the descriptions may be largely limited to those of the rabbit and man.

THE COLLOIDAL STRUCTURE OF THE CYTOPLASM OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

Microdissections of polymorphonuclear leukocytes, under oil-immersion objectives, have shown that the usual descriptions of their structure are entirely erroneous. The cytoplasm of the white cells of all classes of vertebrates has nothing of the nature of a cell membrane. The surfaces of these cells are as completely naked as those of Rhizopods, nor does the living leukocyte cytoplasm contain a spongioplasm and hyaloplasm. The cytoplasm of this cell is a jelly in which are imbedded large numbers of globules. A very thin sur-

1. *Arch. f. exper. Path. u. Pharmacol.*, 1904, 52, p. 242.

2. *Jour. pharmacol. and Exp. Therapy*, 1913, 5, p. 275.

face layer of cytoplasm goes more or less completely into solution when the surface is incised or extensively torn with glass dissecting needles. This change is so rapid and affects such a small mass of the living cytoplasmic jelly that it may be easily overlooked even when the dissections are made under oil-immersion objectives. With this exception the cytoplasm of polymorphonuclear leukocytes does not change rapidly as a result of mechanical injury. If pieces of the living cytoplasmic jelly between the globules are torn out, they are found to be optically homogeneous or monophasic in molar structure. The well-known turbidity of the living polymorphonuclear leukocyte is due to the globular component; the cytoplasm proper is almost transparent and has extremely little scattering action on light. The structures usually termed cytoplasmic granules are of the nature of separation products; they do not grade into the surrounding cytoplasm. In my terminology, such components of the living substance are termed globules or spherules while those that grade into the imbedding substance are termed granules. This is believed to be a fundamental physical distinction. It is almost impossible to free the globules entirely from the remarkably glutinous cytoplasm. The substances that form the globules change their distribution in the different phases of the leukocyte. In one phase, the globules are localized in one small area of cytoplasm. In other phases, the globules vary greatly in size in different parts of the same cell. The structural death-changes are also commonly associated with marked redistribution of the globular material.³ Spermatzoa and the spermatocytes of insects are the only cells, so far dissected, that may be compared in glutinosity to leukocytes. The cytoplasm is soft and may be easily pulled out into long strands which contract considerably when freed. Pieces of the cytoplasmic jelly do not round up when torn out in diluted plasma. Jams made from colorless berries show physical and optical properties that are in many ways similar to those of the cytoplasm of leukocytes.

STRUCTURAL TRANSFORMATIONS OF POLYMORPHONUCLEAR LEUKOCYTES

The citation of an actual experiment may serve to illustrate the nature of the structural transformations observed. A drop of diluted human blood prepared according to the hirudin method was mounted and sealed with vaselin. The preparation was immediately trans-

3. For similar observations see Schilling, *Folia haematol.*, 1908, 6, p. 429.

ferred to a microscope incubator and examined by dark field illumination. Most of the leukocytes extend blunt pseudopods and by this means flow slowly across the field. Inside of fifteen minutes many leukocytes may be observed to undergo a marked surface change. A surface layer of from one to three or more microns in thickness becomes

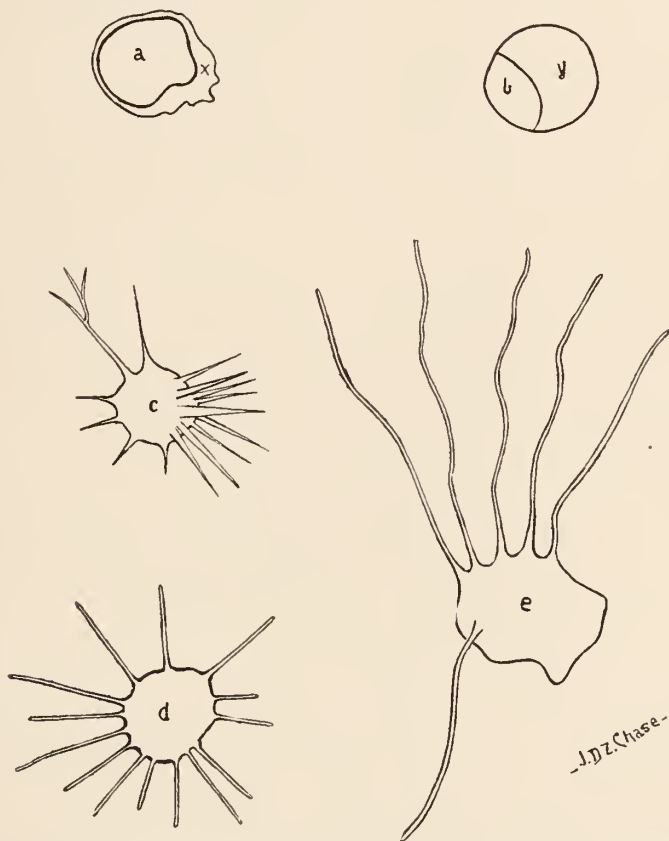


Fig. 1.—Camera drawings of living human polymorphonuclear leukocytes.

a=Hyaline-surface phase; x=Hyaline surface layer; b=Hyaline phase; y=Hyaline area; c=Fine pseudopodial phase; d=Ciliated phase; e=Flagellated phase. The processes usually have an irregular distribution over the surface of the leukocytes, but this important structural detail could not be brought out well in the camera drawings.

All camera drawings were made with 1.8 mm. Zeiss homogeneous immersion objective, No. 6 compensating ocular, and paraboloid condenser.

optically delineated from the underlying substance. This stage is shown in Figure 1, *a*. In less than thirty minutes many leukocytes are seen, covered with delicate protoplasmic processes which may assume

at least three different forms, namely, fine, long immobile pseudopods that frequently branch, short cilium-like processes, and long delicate processes which have the rapid undulatory movement characteristic of flagella. The protrusion and retraction of the different kinds of processes have been observed many times. Leukocytes with as many as twelve or fifteen long undulating processes in one focal plane have been observed to retract all processes in the course of a few seconds and become typical ameboid cells.

The more important distinct forms or phases of the leukocyte are shown diagrammatically in Figures 1 and 2. The hyaline surface phase is characterized by an extraordinary viscosity of the surface. If two leukocytes in this phase are brought in contact, fusion at the

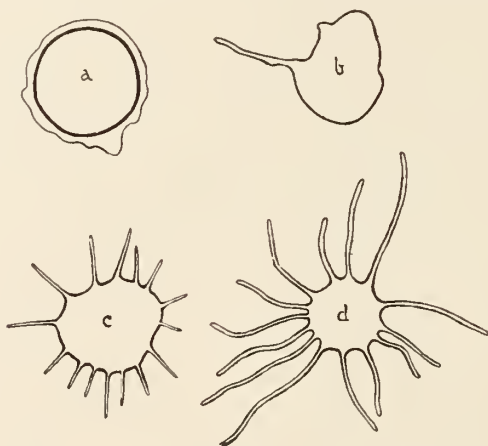


Fig. 2.—Camera drawings of living polymorphonuclear leukocytes of the rabbit.

a = Hyaline-surface phase; b and c = Ciliated phase; d = Flagellated phase.

point of contact occurs. If the surface of such a leukocyte is touched with a glass dissecting needle and the needle drawn away, a minute thread of hyaline cytoplasm may be seen connecting the point of the needle with the leukocyte. When motile bacteria strike this layer they stick and generally do not seem to be able to free themselves. The violent jerking movements made by the bacteria may serve to imbed them firmly in the surface of the leukocytes. Observations of the mesenteries of frogs, exposed to air, indicate that the well-known accumulation of leukocytes on the walls of the small blood-vessels is due, primarily, to a marked increase in viscosity of the surface layers

of the leukocyte cytoplasm. The important power of undergoing rapid variation in surface viscosity, in response to slight environmental changes, is not limited to the white blood-cells of vertebrates, since Hardy⁴ has given an excellent description of this phenomenon in the blood-cells of *Daphnia*.

The pseudopodial phase of a human polymorphonuclear leukocyte is shown in Figure 1. Processes of this type are subject to the widest variation and in some cases reach extraordinary lengths. In 1891 Ranvier⁵ described frog lymphocytes with numerous long pseudopods, under the name of "clasmatocytes." Ranvier considers clasmatocytes to be new types of cells. It appears that retraction of the pseudopods was not observed. More recently Joly⁶ has figured the clasmatocytes of Ranvier.

In the ciliated phase the processes are usually numerous and vary in length from about four to ten microns. The processes exhibit an uncoordinated cilium-like beating. A part of the processes on a cell may be motionless, while others show rapid movement. When the processes reach a length of from ten to thirty or more microns they have the undulatory motion common to flagella. Such forms may be considered flagellated phases. Both the ciliated and flagellated phases may be free-swimming. On account of the lack of coordination in the movements of the processes, such leukocytes follow a very irregular path when moving across the field.

As far as I have been able to determine, both mononuclear and polymorphonuclear white blood-cells of all classes of vertebrates may undergo these strange changes. A peculiar redistribution change that has been frequently observed is characterized by the hyaline character of the greater part of the cell-substance. In this phase the globules are localized in a small area of the cytoplasm.

In this connection two recent papers that have a general bearing on structural transformations in cells may be mentioned. Holmes⁷ has described the transformation of hexagonal peritoneal epithelial cells of the frog into ciliated cells which in turn become ameboid. Holmes writes, "One would not suspect these (ameboid) cells to be derived from ciliated epithelium were it not for the fact that one can actually observe their transformation." In an important study of an ameba

4. *Jour. Physiol.*, 1892, 13, p. 165.

5. *Compt. rend. Acad. d. sc.*, 1891, 112, p. 688.

6. *Arch de méd. expér. et d'anat. path.*, 1898, 10, p. 616.

7. *Science, New Series*, 1914, 39, p. 107.

of the limax group, Wherry⁸ describes the transformation under certain conditions of typical amebae into flagellates. Wherry states that the pressure of a cover glass was generally sufficient to cause the change of the free-swimming flagellated forms back into amebae.

STRUCTURAL CHANGES IN MYELOCYTES

A number of observations have been made on the blood-cells from a case of myelogenous leukemia. Myelocytes with pseudopods and long flagellum-like processes are shown in Figures 1 and 3, Plate 2. In preparations of leukemic blood, prepared by the hirudin method, the myelocytes seemed to be the first cells to extrude processes. Large myelocytes, covered with numerous long undulating processes, make striking microscopic pictures.

In both normal and leukemic blood a small body, about three microns in diameter, containing a few granules and having from one to four long delicate processes, has been observed. The exact identity of this structure has not been determined.

THE BEHAVIOR OF UNATTACHED PROCESSES

Processes that have lost their connection with cells have an independent undulatory movement. A single free process has been observed to retain its characteristic activity unimpaired for over one hour. A process that lies obliquely in the focal plane of an objective actually simulates a spirochete. Such unattached processes seem undoubtedly to be a source of the pseudo-spirochetes of normal blood, so familiar to those investigators who work much with dark field illumination.

It appears certain that active unattached processes have been observed by Rosenthal,⁹ Beer,¹⁰ Chambers,¹¹ Ketron,¹² and others; yet, it should be recalled that preparations made according to the usual methods contain plain and beaded threads of fibrin, which may closely simulate the unattached processes of blood-cells. Besides, Ketron states, "If blood is allowed to flow in a citrate solution to prevent clotting none of these bodies are found and I was not able to produce them by trauma." And further, "These results seem to give definite proof that these pseudo-organisms are most likely products of fibrin formation."

8. *Arch. f. Protistenkunde*, 1913, 31, p. 77.

9. J. Rosenthal, *Festschrift*, Leipzig, 1906.

10. *München. med. Wchnschr.*, 1907, 54, p. 1926.

11. *Lancet*, 1913, 1, p. 1728.

12. *Jour. Cutan. Dis.*, 1914, 32, p. 216.

It may be relevant to note that observers should be able to distinguish between brownian movement about a mean position and the motion exhibited by active living cell processes—either attached or free.

STRUCTURAL CHANGES IN ERYTHROCYTES

A photomicrograph of a living human erythrocyte with several processes is shown in Figure 2, Plate 2. The long process showed typical flagellum-like movement just before the exposure for the photomicrograph was made. The short processes on the same erythrocyte were not well brought out on account of their rapid movement. A red cell with such processes may be actually slowly free-swimming. The processes observed on the erythrocytes of birds, turtles, frogs, and fishes were particularly long and beautiful. As many as fifteen short processes have been seen in one focal plane on a human erythrocyte. Nucleated red cells usually have from one to four very clear or hyaline processes. The protrusion and retraction of the processes have been observed for both nucleated and non-nucleated red corpuscles. Occasionally a long process is completely retracted in much less than a second.

Certain peculiar redistribution phenomena, which occur in sealed preparations of red corpuscles of all classes of vertebrates, are worthy of a brief description. Such structures as granules, rings, curved rods, and undulating filaments, have been seen in the red cells of all vertebrates below mammals. Even the nuclear substance may change its distribution. Changes of the same order, but much less marked, are common in sealed preparations of human red cells. Granules frequently separate out of the corpuscular jelly, and notable changes occur in the distribution and density of the surface layers.

THE CONDITIONS UNDER WHICH VERTEBRATE BLOOD-CELLS UNDERGO STRUCTURAL TRANSFORMATIONS

The conditions under which cell-processes are put out have proved very variable. Human and rabbit white blood-cells, which have remained at room temperature from one and one-half to two hours after removal from the body, usually show all the phases that I have described, when examined in sealed preparations by dark field illumination, at either incubator or room temperature. The change from body to room temperatures seems to have a peculiar effect on the

white cells of birds and mammals. For the first one or two hours after removal from the body, these cells are usually either inactive or show only ameboid movement and the hyaline surface phase. Incubator temperature may greatly shorten the time of appearance of processes. White blood-cells with processes have been observed in pieces of living tubercles from mouse lung; in exudates from corneal tubercles; in the mesenteries of frogs, which had been exposed to the air for several hours; and in samples of pus.

Very little has been made out about the precise conditions under which mammalian red corpuscles and nucleated erythrocytes of the other classes of vertebrates extrude processes. Human red corpuscles at room temperature do not usually show processes for some hours. In one preparation of human blood, kept at room temperature for about four hours, almost every red corpuscle had a single long undulating process. In the course of a thirty-minute observation many of the processes were retracted. The nucleated red cells of pigeons, turtles, frogs, and fishes, when mounted and sealed, and kept at room temperature, showed processes in from three hours to two days or even longer. Many human and nucleated red cells with undulating processes, which had lost their hemaglobin and were almost invisible, were observed. Certainly both nucleated and non-nucleated red corpuscles may be very active after all visible hemaglobin is lost. A decrease in oxygen tension appears to be a factor of some importance in the production of transformations in white and red blood-cells. I have recently succeeded in demonstrating the presence of numerous processes on white blood-cells which were mounted in shallow drops in a moist chamber and left for one hour at room temperature and then subjected to a temperature of 0 C. and fixed at this temperature with osmic vapor.

THE EFFECT OF LOW OXYGEN PRESSURE ON THE ACTIVITY OF BLOOD-CELLS

An extensive usage of sealed preparations has brought out the important fact that the white and red cells of vertebrates have the power of adjusting their metabolism to very low partial pressures of oxygen and even to anaerobic conditions. Very active red and white cells have been observed in sealed preparations kept at room temperature for three days.

THE RELATION OF STRUCTURAL CHANGES IN LEUKOCYTES
TO PHAGOCYTOSIS

A number of experiments have been made with the aim of gaining if possible a clearer insight into the mechanism of the phagocytosis of bacteria by leukocytes and of determining the exact form taken by leukocytes in conditions similar to those of the usual in-vitro methods of studying phagocytosis. The results are of considerable interest. Leukocytes prepared according to the opsonic technic, mounted and sealed, and examined on a dark field in a microscope incubator, usually show the hyaline surface change in from ten to twenty minutes. White blood-cells in this phase frequently exhibit a rapid oscillatory flowing of parts of the surface jelly. The movement of globules in this layer has been frequently misinterpreted by previous observers and considered brownian movement. Leukocytes with numerous moving processes have been observed in such preparations in less than ten minutes after their removal to the incubator. The addition of emulsions of staphylococci to leukocytes does not change their behavior in any way that I could determine. In preparations of mixtures of motile bacteria and white blood-cells containing ciliated or flagellated forms, the actual entanglement of the bacteria by the processes was observed a great number of times. Occasionally the violent struggle of a bacterium resulted in freeing it from the leukocyte instead of more thoroughly imbedding it in the surfaces of the processes, as usually seemed to be the case. Further, the results of all my experiments on in-vitro phagocytosis point to the varying viscosity of the surface layers of the white cells as a fundamental factor in the fixing of bacteria to their surfaces. The oscillatory flowing of parts of the cytoplasm may well prove to be capable of carrying bacteria into the deeper layers of the leukocyte.

Finally it seems well to emphasize some of the optical difficulties which have been met in the course of this investigation. It is a well-known principle of physical optics that transparent bodies, which have the same refractive index and dispersive power as the mounting medium, are invisible when viewed by transmitted light. This important optical principle holds for many of the structural changes in blood-cells that I have described. Redistribution of the globular substance is about all that can be seen with achromatic objectives and uncorrected condensers. With the notable exception of cilium- and

flagellum-like processes, about all the structural transformations may be observed by transmitted light with the optical system described above.

All the structural changes in blood-cells that I have figured or described may be observed with dark field illumination, yet this method has only a qualitative value. Processes on the upper and lower surfaces of cells are usually invisible. The long hyaline processes are likewise invisible unless the background is intensely black and they lie well within the focal plane. Another serious limitation of dark field illumination is the frequent failure of this optical method to bring out the exact structural relations of rapidly moving processes to the cell body. This particular limitation seems to be general, since an examination of numerous ciliates and flagellates gave similar results.

I am indebted to Dr. Paul A. Lewis for helpful criticisms and to Prof. C. E. McClung for the photographs.

EXPLANATION OF PLATE 2

Fig. 1.—Photomicrograph of blood-cells from a case of myelogenous leukemia.

Blood-cells prepared by the hirudin method, and fixed wet, at about 0° C. with osmic vapor and stained with iron hematoxylin. Myelocyte showing three long delicate processes. Magnified about 1000 diameters.

Fig. 2.—Photomicrograph of living human erythrocytes on dark field.

An erythrocyte with one long and several short processes. Magnified about 1000 diameters.

Fig. 3.—Drawing of myelocytes.

a=A large myelocyte covered with fine pseudopods; b=A medium-sized myelocyte with long delicate processes of the flagellum type. The granules on two of the processes are probably artefacts, as such structures were not observed in living, active myelocytes. The leukemic blood was prepared by the hirudin method and fixed wet, at low temperature, with osmic vapor and stained with iron hematoxylin.

Magnified about 750 diameters.

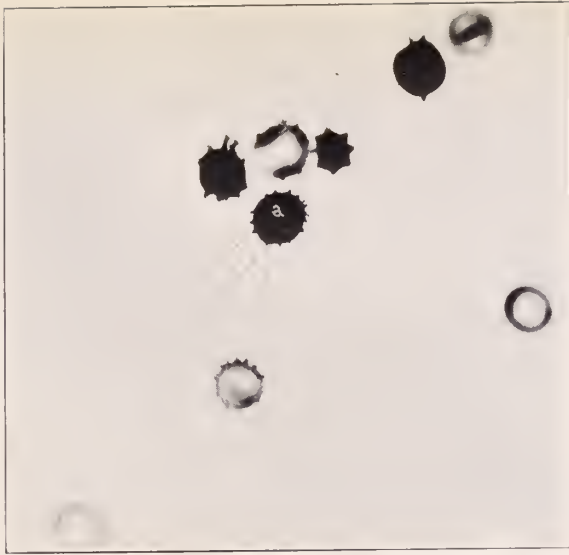


Figure 1

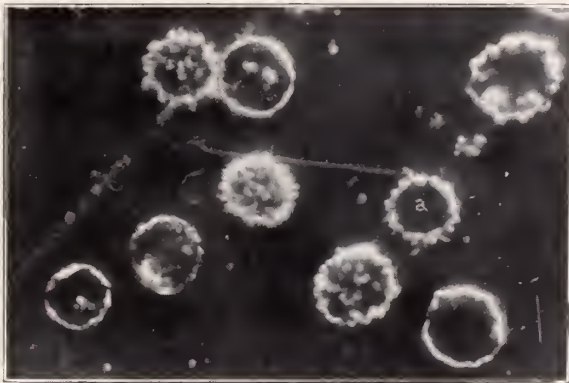


Figure 2

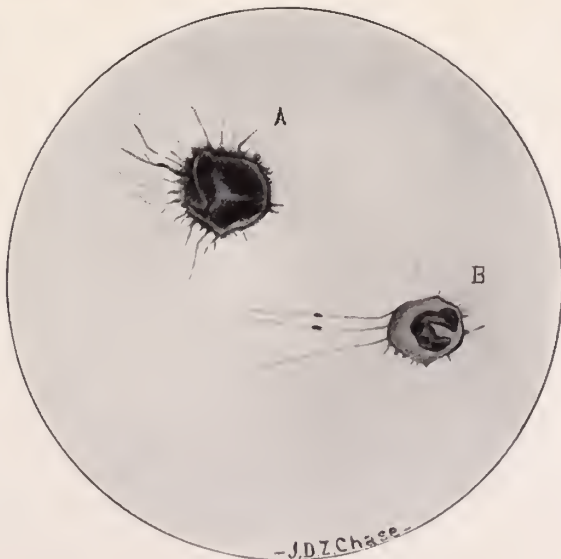


Figure 3

INFECTION OF MAN WITH BACTERIUM TULARENSE *

WILLIAM B. WHERRY AND B. H. LAMB

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PLATE 3

We believe this to be the first recorded instance of human infection with the organism discovered by McCoy and Chapin in a plague-like disease first described by McCoy in the California ground-squirrel (*Citellus beecheyi* Richardson).¹ In our work we have added very little that is new connected with the biology of the organism, besides the fact that our virus came from a human being who in all probability contracted it while dressing meats for a restaurant. In this connection we might remark that about a year previously we had heard from a hunter that wild rabbits were dying in large numbers across the Ohio River in Kentucky. We did not see any of these rodents, and by the time we had worked out the etiology of our case it was too late to investigate wild game sold in the markets. For the fact that our own work was carried out to a satisfactory conclusion in a relatively short time, we are indebted to the work of McCoy and Chapin, whose labors covered a considerable period; for we did not succeed in cultivating the virus until we used the coagulated egg-yolk recommended by them. While we occasionally refer to some of the extensive experiments of these workers, we do not review them in detail, as the publications referred to are easily obtained.

HISTORY OF CASE²

On Nov. 24, 1913, the patient, E. E., a man of 21 years, who was meat cutter in a restaurant, came, presenting an inflammation of the left eye which he said started on November 21. On examination about ten discrete ulcers, varying from 1 to 4 mm. in diameter, were seen on the conjunctiva covering the upper and lower tarsi. The lids were edematous. The preauricular gland on the left side was swollen and tender. During the next three days the edema increased, the

* Received for publication, June 6, 1914.

1. *Public Health Bull.* 43, U. S. *Public Health Service*, Washington, 1911; *ibid.*, No. 53, 1912; and *Jour. Infect. Dis.*, 1912, 10, p. 61.

2. We are indebted to Dr. Derrick T. Vail of Cincinnati for the greater part of this clinical history.

ulceration became a little more marked, and the lymphatic glands in the left anterior triangle of the neck and the left submaxillary glands became tender and swollen. On November 28, a week after the onset, the patient's temperature was 101.6 F.; he looked cachectic and complained of losing weight. A discrete pustular eruption now appeared on the left temporal and malar region. Glanders was suspected and the patient was sent to the Cincinnati hospital. By December 1, in addition to the above symptoms, which persisted, the inflammatory process spread to the left lacrimal sac and resulted in abscess formation. At this time smears and cultures on various media were made. No bacteria could be found microscopically, nor did any growth appear on the culture media during about a month's observation. Scrapings from one of the ulcers were injected into a male guinea-pig as follows:

Guinea-Pig 1.—Received on Dec. 4, 1913, an intraperitoneal injection of scrapings from a conjunctival ulcer suspended in sterile physiologic salt solution. Died, December 10. Kept on ice over night. Necropsy, December 11: The body is frozen solid. On section the subcutis and superficial glands appear normal. The lungs show acute pneumonia, the spleen and liver are congested and enlarged, and show numerous scattered foci of necrosis. Smears from the heart's blood and various organs were stained by Giemsa's method, with Löffler's methylene-blue, by Gram's and by the tubercle method, and careful search failed to reveal anything looking like bacteria excepting that in the Giemsa preparations very minute scattered coccoid granules were seen. Both aerobic and anaerobic cultures made on a variety of media remained sterile at 37 C. and at room temperature during a month's observation.

The patient left the hospital still suffering from the disease, and further efforts to get material from him for confirmatory inoculations failed. There can hardly be any doubt, however, about the virus coming from the human case, since we have been able to reproduce the gross changes in the conjunctivae so accurately in experimental animals.

ISOLATION OF THE VIRUS

The disease was kept going through a series of guinea-pigs while experiments on isolation were in progress. Aerobic and anaerobic cultures were made in various media, e. g., +1 agar; agar with glycerin; with glucose, maltose and other sugars; with human blood, ascites fluid, defibrinated rabbit's, rat's and guinea-pig's blood; Löffler's blood-serum; in +1 broth containing sterile pieces of guinea-pig's liver, spleen and kidney (with and without a covering of oil). In all instances the cultures remained sterile excepting that once a bacillus with a tendency to produce capsules, and another time

a diplococcus, was grown. Neither of these was pathogenic for guinea-pigs. All the cultures were kept under observation for about a month before they were discarded. The following inoculations show that the virus did not live for long in some of these cultures.

Guinea-Pig 16.—Inoculated subcutaneously with 1 c.c. of a forty-eight-hour culture (37 C.) from White Rat 4—made by placing one-half of its spleen in a tube of +1 broth containing a piece of fresh guinea-pig's liver and covering the surface with sterile olive oil. A minute streptococcus was present in the culture. The guinea-pig appeared healthy for six days, but was chloroformed in a dying condition on the seventh. No signs of the disease were present and cultures from its heart's blood remained sterile.

Guinea-Pig 18.—Inoculated subcutaneously with 1.5 c.c. of a six-day culture (37 C.) from Rabbit 1—made by placing a bit of the rabbit's spleen in +1 broth containing a fresh piece of the kidney of a guinea-pig and covering with sterile olive oil. The guinea-pig remained well during twenty days' observation, when it was reused successfully for a passage of the virus.

After we had passed the virus through twenty-four animals, we became acquainted with the work of McCoy and Chapin, and by using the coagulated egg-yolk on which they were able to grow *Bacillus tularense* we isolated what we believe to be the same bacterium.

During these attempts at cultivation we made careful studies of fresh preparations and smears from the organs of dead and freshly killed animals. The smears were fixed by heat, with methyl alcohol, with Zenker's or Schaudinn's fluid and stained over short and long intervals with Löffler's methylene-blue or carbol fuchsin, with Giemsa's and McNeal's stains, by Gram's method and aqueous safranin and with Heidenhain's iron-alum hematoxylin. Vital staining with various anilin dyes was tried. The preparations were studied carefully with apochromatic lenses and compensating oculars without revealing anything we could identify as a probable micro-organism. The following two filtration experiments showed that the virus would not pass a Berkefeld filter.

(a) Guinea-Pig 4 was chloroformed when dying and 0.5 c.c. of its viscous peritoneal exudate was diluted with 20 c.c. of 0.85 per cent sodium chlorid solution and filtered through a new No. 5 Berkefeld filter. Guinea-Pig 6, 380 gm., was inoculated intraperitoneally with 10 c.c. of the filtrate. It remained well for twenty-three days, when it died for some unknown reason.

(b) The enlarged speckled spleen of Rabbit 3, dead of the disease, was kept on ice in a sterile Petri dish for forty-eight hours. It was examined and found microscopically sterile. Half of the spleen was ground up in a mortar and suspended in 40 c.c. of sterile 0.85 per cent sodium chlorid solution. After sedimenting for one-half hour, the supernatant fluid was filtered through the same No. 5 Berkefeld filter used in (a). The filter was boiled thoroughly in sodium carbonate solution, washed with distilled water until the filtrate was

no longer alkaline to phenolphthalein, and then sterilized in the autoclave. It took fifteen minutes at ordinary pressure to filter 20 c.c. of perfectly clear, straw-colored fluid. Guinea-Pig 19, 290 gm., inoculated intraperitoneally with 6 c.c. of this filtrate remained well for nine days, when it was found dying with spasmodic convulsions. On dissection it showed none of the lesions of the disease and another guinea-pig (No. 26, 350 gm.) inoculated intraperitoneally with 2 c.c. of its peritoneal exudate and heart's blood remained well for nineteen days, when it was killed, dissected and found normal.

Guinea-Pig 20 (365 gm.), used as a control, was injected subcutaneously with 2 c.c. of the unfiltered extract. It died in three days with typical lesions, and *B. tularensis* was isolated in an ovomucoid-yolk culture from an inguinal bubo.

These experiments agree with those of McCoy and Chapin. Since the virus would not pass the Berkefeld No. 5, which allows some clearly visible bacteria to go through, as was shown by one of us in repeating some work done previously by Theobald Smith on the bacillus of guinea-pig pneumonia,³ we felt that probably it was not ultra-microscopic. The virus described below is just as small as, if not smaller than, that of guinea-pig pneumonia, so that its failure to pass the filter may possibly be due to the presence of the large amount of capsular substance.

We examined preparations from the conjunctival ulcers and spleen of an infected rabbit, dead for from six to eight hours, in the dark field. This revealed nothing except the presence of very numerous minute granules with active brownian motion, and similar colloidal granules could be found in smaller numbers in the spleen of a normal rabbit. In all probability some of these granules were *B. tularensis* which assumes a coccoidal form post mortem, but they could not be distinguished from other colloidal granules.

DESCRIPTION OF THE VIRUS AND ITS IDENTITY WITH BACILLUS TULARENSIS

After we had obtained cultures of *B. tularensis* from some of our animals, we found by experimenting that while carbolic acid and caustic potash were inefficient mordants, anilin oil gave excellent results with the cultures and with tissue smears. We have obtained the clearest pictures with anilin-water Hoffman's violet or Victoria blau; anilin-water fuchsin, dahlia and gentian violet also stain the bacilli in smears, but they are not differentiated as clearly; anilin-water nacht blau and toluidin blau failed to stain the bacilli even after fifteen to thirty minutes.

3. *Jour. Med. Research*, 1902, 3, p. 322.

As an example of this selective staining we may quote the following:

Guinea-Pig 27 (480 gm.) was autopsied some hours after death and a culture from its spleen on egg-yolk showed a spreading, moist growth which is not characteristic of *B. tularense*. Smears showed enormous numbers of bacilli which stained readily with Löffler's methylene-blue, but when the smears were stained with anilin Hoffman's violet, enormous numbers of minute coccoid bodies were also seen among the rods.

B. tularense does not retain the stain in Gram's method, nor will it take a contrast stain of aqueous safranin in such preparations. While McCoy and Chapin state that it is stained with anilin gentian violet and carbol fuchsin, we have not been able to demonstrate it clearly with the latter dye in tissue smears, although this stain does fairly well for smears from cultures, where, in our experience, the capsular substance is greatly diminished in amount.

It is a minute rod surrounded by a considerable amount of capsular substance when in the tissues of infected animals. We have not tried to stain this capsule in smears, but it is apparent as a very well-defined, unstained halo. In our experience the rod shape is only to be seen when an animal is chloroformed before death. Post mortem, the organism soon rounds up into coccoid bodies, and this latter form is the only one we have seen in coagulated egg-yolk or yolk-ovomucoid cultures. No distinct capsule is to be seen in cultures. When measured under a Zeiss 3 mm. N. A. 1.40, the coccoid form from egg-yolk cultures measures about 0.5 to 0.6 micron in diameter. The rods, including the capsular halo, measured about 1 to 1.5 microns \times 0.7 micron. We will not insist on the accuracy of these measurements, however, as an ocular micrometer was used.

McCoy and Chapin give the following approximate measurements for *B. tularense*: length, 0.3 to 0.7 micron; length of capsule, 0.4 to 1 micron; breadth, 0.2 micron; breadth of capsule, 0.3 to 0.5 micron. They also note that coccoid forms occur in the tissues and that irregular and globular forms predominate in cultures.

Cultural Characters.—We have obtained growths from the spleen, liver, buboes and heart's blood of animals on the coagulated hen's egg-yolk, recommended by McCoy and Chapin. We can confirm their observation concerning the importance of having the egg-yolk of just the right consistency. The medium must be of a rubbing consistency, so that it fluctuates when touched, and yet the surface must be firm enough for inoculation. Further solidification beyond this point, as usually practiced in making Dorset's medium, renders the

medium unfit for the use of the bacterium. McCoy and Chapin recommend diluting the egg-yolk with 20 per cent. of water or milk. We have taken the yolk of perfectly fresh hen's eggs and tubed it after diluting it with an equal quantity of sterile 0.85 per cent. sodium chlorid solution. The whole procedure is carried out with aseptic precautions. The slanted tubes are then coagulated at from 70 to 90 C., being taken out of the sterilizer when they have attained just the right consistency.

On such slants, inoculated from the tissues of experimental animals and incubated aerobically at 37 C., growth appears on the second or third day. The colonies are very minute at first, but reach a diameter of from 1 to 3 mm. They appear like minute drops of ovomucoid. They are viscous and emulsify with difficulty in salt solution. When removed with precautions to avoid taking up yolk granules from beneath, and examined in hanging drops of 0.85 per cent. sodium chlorid solution or broth, they appear as minute coccoid bodies. We have watched them for hours under the Zeiss 3 mm. N. A. 1.40 with compensating oculars, and feel sure that under these conditions of oxygen tension they show no motility excepting active brownian motion. The coccoid bodies are discrete, and but rarely can one find two which are in juxtaposition. As one might expect, actual fission has not been observed under these circumstances. Egg-yolk cultures have been found viable after three months at 15 C. A culture isolated on January 27 and subcultured every third or fourth day until March 24, was found virulent for Guinea-Pig 49, but produced a subacute form of the disease.

All attempts to accustom this organism to other media, even after several weeks' cultivation on egg-yolk, have failed except that we found that it would grow in the ovomucoid-yolk medium recommended by one of us for the cultivation of amebae.⁴ Here the fluid medium showed no visible change except that in three or four days after inoculation with a small piece of an inguinal bubo (guinea-pig) the medium solidified so that the tube could be turned upside down without spilling the culture—just as happens when this medium is inoculated with *Bacillus pyocyaneus*. Evidently something like pseudo-mucin is produced, and the production of some such substance probably accounts for the viscosity of the colonies and the viscosity of the peritoneal exudates in experimental animals. Subcultures in the

4. *Arch. f. Protistenk.*, 1913, 30, p. 77.

same medium gave the same results at 37 C. Here, too, the organism occurs in the coccoid form.

Pathogenicity.—We have not experimented on animals as extensively as McCoy and Chapin, but have used forty-nine guinea-pigs, three Belgian hares, three white rats, three kittens and one pigeon. The guinea-pigs, as a rule, succumbed on the fourth or fifth day after cutaneous inoculation with spleen juice or when pricked in the eye with an infected needle. In a series of twelve passages (including one rabbit), where the virus was passed from the spleen of a dead animal to the conjunctiva or cutis of a living one, there was no marked diminution or exaltation of virulence.

As an example of the usual lesions in guinea-pigs, we quote the following protocol of Guinea-Pig 3 (439 gm.): Inoculated cutaneously by rubbing a fragment of the spleen of Guinea-Pig 2 on a scratch on its abdomen. Three days later the site of inoculation showed an area of congestion. In the center of this area was a yellowish dry, crusty scab beneath which there was no fluid pus. The animal was very sick and weak. It died on the night of the fourth day. At the postmortem next morning the site of inoculation showed a yellowish area of coagulation necrosis, 1 by 2 inches, surrounded by congested vessels which radiated especially to the inguinal glands. These glands were swollen, 3 by 4 mm., were hard and not caseous on section, and were surrounded by intensely congested and edematous tissue. The liver and spleen were swollen and enlarged and speckled with numerous yellowish-white foci of necrosis. The kidneys were intensely congested. The adrenals were swollen and so congested as to appear blackish and were as friable as a clot of blood. The mesenteric glands were swollen and showed a few foci of necrosis on section. The intestines and their follicles appeared normal. The mucous membrane of the stomach appeared normal, but the organ was full of a clear viscous mucus. The epididymi were congested. The vessels of the head were deeply congested and the heart was full of dark-clotted blood. The lungs were congested and showed some small areas of consolidation and a few subserous petechiae. The brain appeared normal to the eye.

The hemorrhagic congestion of the adrenals is not always so marked. The peritoneal cavity often contains a small amount of extremely viscous fluid. Once we noted very marked involvement of the lymph follicles of the small intestine with intestinal subserous hemorrhages in a guinea-pig inoculated cutaneously. It appears to us, on comparing our notes with those of McCoy and Chapin, that we were dealing with a somewhat more virulent virus, as shown by the somewhat shorter course of the disease in our animals and the absence of caseation of the buboes which they note as characteristic.

We kept the virus going chiefly by rubbing a little spleen pulp into a scratch on the abdomen of animals. Simply dipping a fine needle into the spleen of a dead animal or into a culture, and pricking the

ocular or palpebral conjunctiva of rabbits or guinea-pigs results in the production of multiple areas of necrosis on the palpebral conjunctivae, just like those in the human case, and is followed by septicemia and death within a very few days.

The rabbits formed an eye inoculation series—No. 2 being inoculated from the spleen of No. 1, and No. 3 from the spleen of No. 2. Death occurred on the sixth, third and fourth days, respectively.

As an example of the lesions in rabbits, we may quote the protocol of Rabbit 1. The post-mortem appearance of the eye, spleen and liver of this animal are reproduced in Plate 2, Figures 1, 2 and 3.

Rabbit 1.—(Full grown Belgian hare, weight not recorded.) On December 13 pricked the conjunctiva of the upper lid of the right eye with a fine needle dipped into the spleen pulp of Guinea-Pig 2. On the third day thereafter the eye was seen to discharge an abundant purulent exudate and the upper lid was congested and edematous. On the fourth day the eyelids which were glued together were separated and the pus washed out with physiologic salt solution. The conjunctiva of the much-congested edematous upper lid showed four discrete yellowish-white areas of necrosis, 2 to 3 mm. in diameter, from which the necrotic material could not be removed by rubbing with a cotton swab. Death occurred during the night of the sixth day.

Post-mortem Examination: The right eye showed considerable congestion and swelling of both lids; the upper and lower palpebral conjunctivae showed a number of pale yellowish-white, irregularly contoured areas of necrosis which were raised above the level of the mucosa, giving the appearance of an adherent membrane; the subcutaneous tissues along the course of the jugular vein on the same side were congested and the lymph-nodes here were enlarged and presented congested capsules. One of the glands was about 8 by 4 mm. in size and on section was of a hard, fibrous consistency, and the cut surface appeared whitish; there was no free fluid in the thoracic and abdominal cavities; the spleen and liver were greatly enlarged, congested, of a firm consistency and full of yellowish-white areas, irregular or nodular, varying from 0.5 to 1 to 2 mm. in diameter; the intestines and abdominal glands appeared normal; the kidneys were congested; the vessels of the heart were deeply injected, and the lungs showed numerous patchy areas of congestion.

The three white rats were inoculated intraperitoneally with guinea-pig spleen extracts and died in two days or less, showing marked injection of the subcutaneous tissues and congestion of the liver, and such congestion of the spleen as to give it a blackish color. In one instance there was also present a complete double pneumonia.

The kittens, which weighed about 400 gm. each, received guinea-pig spleen juice by the ocular, cutaneous and subcutaneous methods, but remained well during several weeks' observation.

The pigeon was inoculated subcutaneously with an emulsion of guinea-pig spleen. It survived during two months' observation, but

as the control showed that the virus was dead (48 hours old), this experiment was worthless.

Some experiments showed that death might occur when infectious material is simply placed on the uninjured mucous membrane of the eye or nose, although the first experiment yielded negative results:

On the fourth day after the inoculation of Rabbit 1 some pus was taken from its eye on a cotton swab and rubbed into the left conjunctiva of Guinea-Pig 8. The eye appeared perfectly normal for three days when it was pricked with a needle dipped in the spleen of the same rabbit. A twenty-four hours infection was manifest and death occurred on the fifth day. The post mortem showed characteristic lesions throughout and very similar to those described above for Rabbit 1.

A few drops of an emulsion from the spleen of Guinea-Pig 33, dead of the disease, were allowed to fall into the left eye of Guinea-Pig 38; the latter died in four days, and at post mortem multiple small discrete areas of necrosis were present in the upper and lower conjunctivae; the jugular glands on the corresponding side of the neck were involved and the lesions in the spleen and liver were typical.

A few drops of the same emulsion from Guinea-Pig 33 were allowed to fall into the left nostril of Guinea-Pig 37; the latter was found dead on the third day thereafter; there was a purulent exudate in the left eye, but no ulcers; the jugular glands, spleen and liver showed characteristic lesions.

Single experiments on transmission by feeding and by cohabitation resulted positively in the former case and negatively in the latter:

Guinea-Pig 39 ate most of the spleen of Guinea-Pig 33, which was chopped up and mixed with bread. It died in three days and showed characteristic changes in the liver and spleen and involvement of the cervical glands. The lymph follicles in the small and large intestines showed marked congestion and hypertrophy.

Guinea-Pig 36 was placed in the cage containing Guinea-Pig 35, which was ill of the disease. The latter died one day later, so No. 36 was transferred to the cage containing No. 41, which was ill of the disease. In two days No. 41 died and was allowed to remain in the cage with No. 36 for three days, when the latter was transferred to a fresh cage. Eleven days later No. 36 died without developing any characteristic lesions.

It will be seen that our experimental inoculations, as far as they go, correspond with the findings of McCoy and Chapin. They found guinea-pigs, rabbits, white rats, gray mice, ground-squirrels (*C. beecheyi*), the gopher (*T. bottai*), the Java and Rhesus monkeys quite susceptible; while the adult Norway rat (*M. Norvegicus*) was usually immune; the young were only partly resistant; sheep were only slightly susceptible (one out of five). The following animals they found immune: calf, pig, goat, cat, dog, pigeon. They were able to transmit the disease to guinea-pigs and ground-squirrels by feeding and by nasal inoculation. They found that 0.000001 c.c. of guinea-pig's

blood was sufficient to convey the disease to another guinea-pig. They found that the disease was not transmitted by contact or association under the conditions of their experiments, but in searching for a probable mode of natural transmission they twice successfully transmitted the disease by means of 100 and 500 squirrel fleas (*Ceratophyllus acutus*), respectively. They found that the virus in tissues on ice dies in four or five days, and when dried in the incubator for forty-eight hours. We noted about the same degree of longevity on ice and found the virus innocuous when dried at room temperature over calcium chlorid. McCoy and Chapin showed that the disease was not related to plague, for a guinea-pig immune to a virulent culture of *B. pestis* promptly succumbed to this virus.

SUMMARY AND CONCLUSIONS

* A case of ulcerative conjunctivitis and lymphadenitis in man is shown to be caused by a minute, capsulated bacterium in all probability identical with *B. tularensis*, which was first discovered by McCoy and Chapin in a plague-like disease of the California ground-squirrel (*Citellus beecheyi* Richardson). Without the employment of very special methods of staining and cultivation, the virus, although it was known not to pass the Berkefeld filter No. 5, remained invisible. We recommend anilin-water-Hoffman's violet particularly for its demonstration, and in addition to the coagulated hen's egg-yolk recommended by McCoy and Chapin for its cultivation, we find that hen's ovomucoid with a trace of yolk is also a favorable medium. Our findings would seem to indicate that this disease is wide-spread among rodents. Further, we wish to call attention to the fact that this recently discovered disease of rodents is apparently sufficiently virulent for gray mice (*Mus musculus*) to warrant the presumption that it may some day take its place along with *B. pestis* as a menace to man.

EXPLANATION OF PLATE 3

Figs. 1, 2 and 3 show the gross appearance of the conjunctivae, liver, and spleen of Rabbit 1. Figures 4 and 5 show the spleen and hard, white, inguinal bubo (surrounding congestion not well preserved) of a guinea-pig. All figures natural size. For descriptions see text.

Fig. 6.—Smear from the spleen of a guinea-pig which was chloroformed just before death. It shows both the rod and coccoid forms surrounded by the capsular halo. $\times 2000$. Anilin Hoffman's violet.

Fig. 7.—Smear from a 3-day-old culture of *B. tularensis* on coagulated egg-yolk. Anilin fuchsin. $\times 1000$. Photomicrographs by Dr. Chas. Goosmann.

PLATE 3



Figure 1

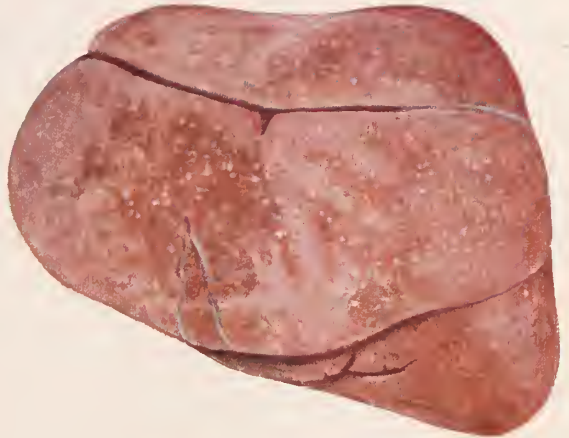


Figure 2



Figure 3

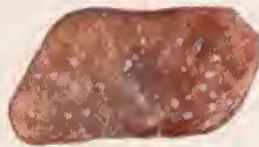


Figure 4

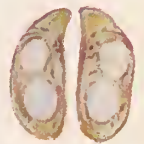


Figure 5

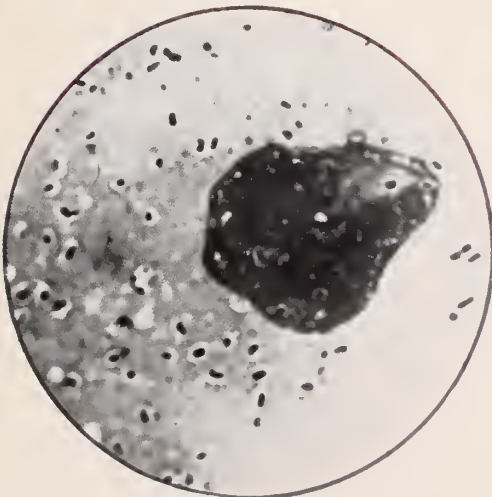


Figure 6

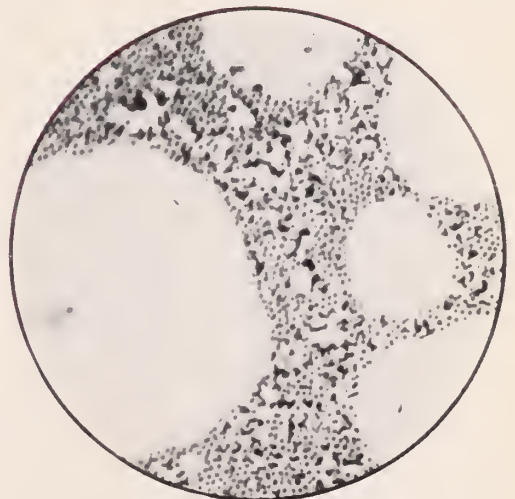


Figure 7

THE ETIOLOGY OF DENGUE

AN ATTEMPT TO PRODUCE THE DISEASE IN THE RHESUS MONKEY BY THE INOCULATION OF DEFIBRINATED BLOOD*

C. H. LAVINDER AND EDWARD FRANCIS

(United States Public Health Service)

It is our purpose somewhat briefly to report experimental work done by us at the United States Marine Hospital, Savannah, Ga., during October and November, 1913, on the attempted transmission of dengue fever to the rhesus monkey by the inoculation of fresh defibrinated blood drawn from cases of the disease, which at that time was epidemic in the city.

Dengue is a disease of special interest to the public health officer on account of its close resemblance epidemiologically and clinically to yellow fever. To the investigator it also offers an inviting field, since the present status of its etiology is such that it apparently offers results of real worth for the expenditure of some time, money and energy.

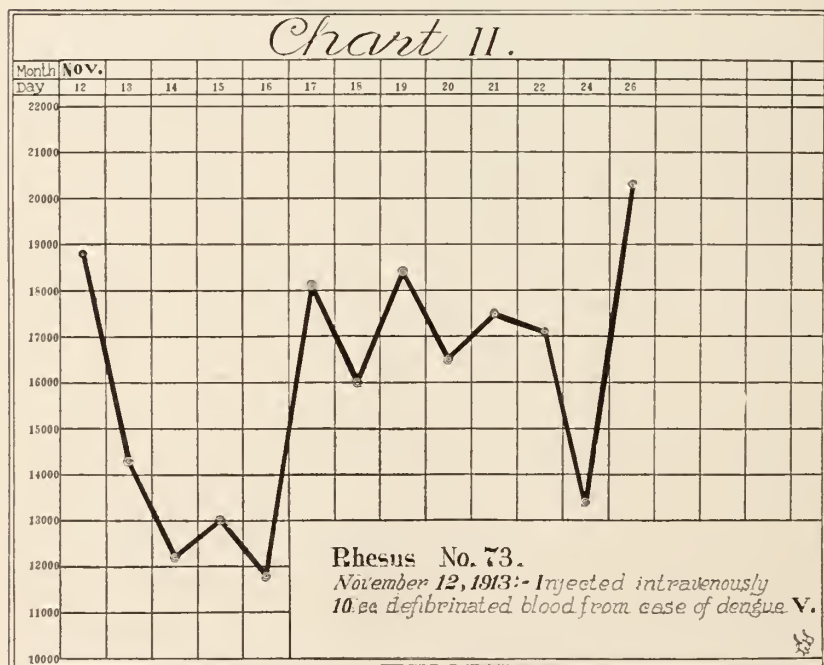
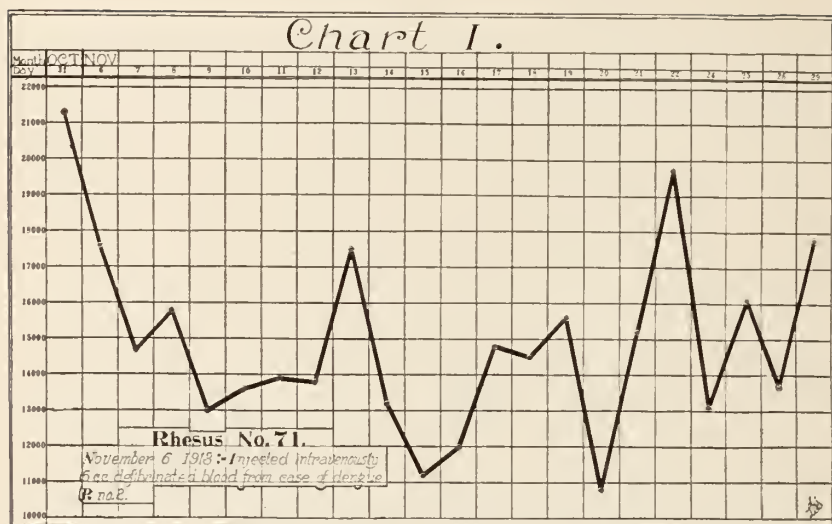
From a review of the subject it would seem safe to assert that dengue is an infectious disease, but is not contagious, that the specific organism is found in the peripheral blood-stream of patients suffering from the disease (at least on the third and fourth days), that the virus is filterable, that no micro-organism of etiological significance has been found in the blood either by staining or by cultural methods, and that the disease is possibly transmitted by the mosquito (*Culex fatigans* and *Stegomyia calopus*). We could find no record in the literature that the disease had been reproduced in animals, and no record of any attempt to reproduce it in the monkey or in the higher apes.

Most, if not all, of our definite knowledge regarding the etiology of dengue rests on the work of Graham¹ and Ashburn and Craig,² especially the latter authors. Their work should, however, be carefully confirmed, especially with regard to mosquito transmission of the disease. Indeed, in its present status, the theory of mosquito transmission of dengue can hardly withstand strict scientific scrutiny.

* Received for publication, June 20, 1914.

1. *Jour. Trop. Med.*, 1903, 6, p. 209.

2. *Philippine Jour. Sc., B*, 1907, 2, p. 93.



Its confirmation should be undertaken and the matter definitely determined. Our work, however, was exclusively an attempt to reproduce the disease in the monkey by the inoculation of defibrinated blood.

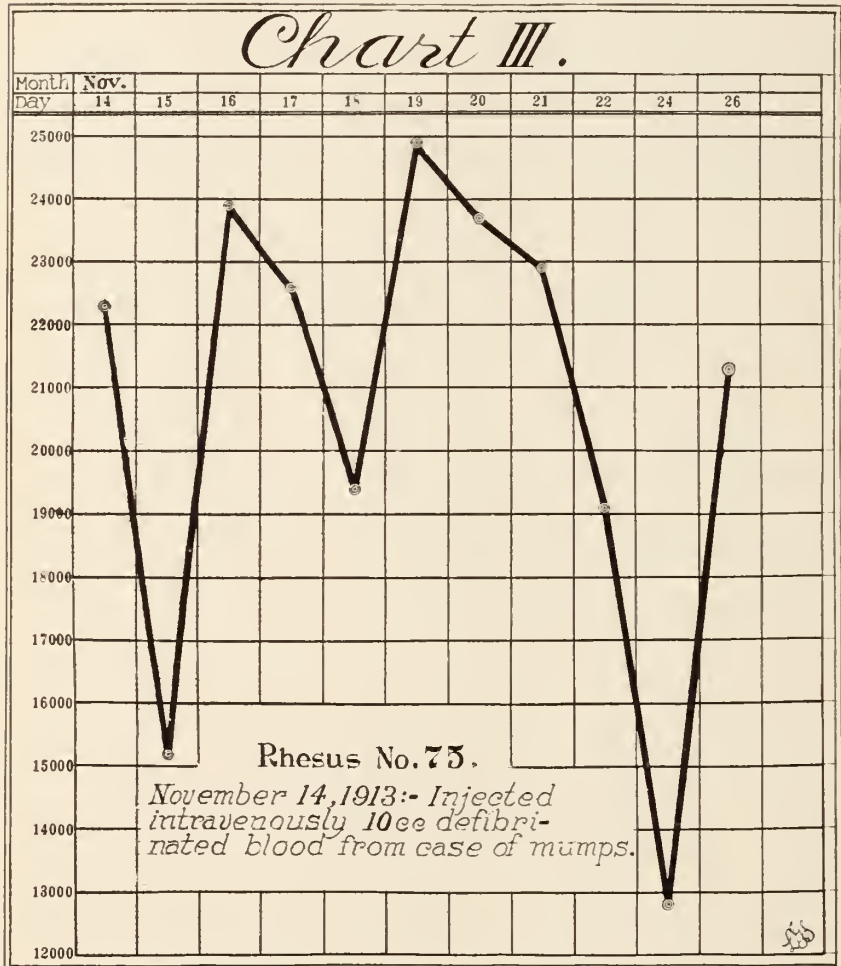
To this end we secured blood from the arm vein of cases of dengue in various stages of the disease, and after defibrination, this was injected intravenously and subcutaneously into monkeys, all as shown in the table given below. The blood was drawn at the residence of the patient with a glass syringe, expelled into a large tube, defibrinated by carefully stirring with a sterile glass rod, brought at once to the laboratory and put in the ice chest until used. For use it was filtered through gauze to get rid of clots. All the monkeys were fresh, healthy, young animals.

TABLE 1
SHOWING CASES OF DENGUE AND ANIMALS INOCULATED

Cases	Quantity Blood Injected	Remarks
E.—4.5th day illness, in eruption	10 c.c. intravenously; 3 c.c. intravenously	Rhesus 9; rhesus 14; Same blood kept 24 hours in ice chest
R.—3.4th day illness	8 c.c. intravenously	Rhesus 10
McA.—2.3d day illness	6 c.c. intravenously	Rhesus 16
S.—4th day illness, in eruption (?)	6.5 c.c. intravenously	Rhesus 23
W.—2d day illness	5 c.c. intravenously; 1 c.c. subcutaneously	Rhesus 25
F.—5th day illness, in eruption, white blood cells 3,000	5 c.c. intravenously; 1 c.c. subcutaneously	Rhesus 24
R2.—3.4th day illness, in eruption; white blood cells 5,900	6 c.c. intravenously	Rhesus 71
V.—3d day illness, in eruption; white blood cells 8,200	10 c.c. intravenously	Rhesus 73

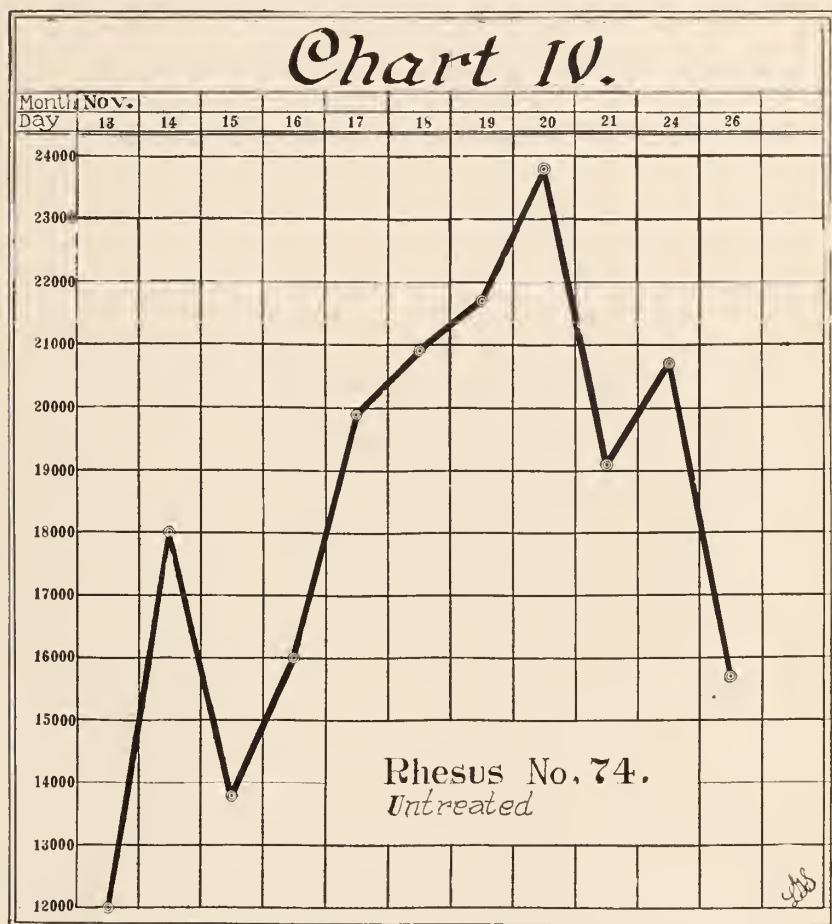
To summarize, it will be seen from the above that we injected intravenously and subcutaneously from 3 to 10 c.c. of defibrinated blood from each of eight cases of dengue into nine rhesus monkeys. The blood was used within two to six hours after withdrawal in all cases but one, where it was kept twenty-four hours in the ice chest. The cases from which the blood was drawn were all carefully selected and in several of them the diagnosis was confirmed by the presence of the characteristic eruption at the time of bleeding. All the animals were carefully examined each day for illness of any kind and for skin eruptions for a period of two weeks. During the same period the temperatures of all were recorded twice daily. They all remained well throughout this period of time and showed no significant deviations of temperature, nor was there any significant skin eruption observed in any of them.

Since leukopenia has been shown to be constant and definite in dengue, it occurred to us that the monkeys might show a similar reaction to the virus, but it was then too late in the epidemic to devote much attention to this. We did, however, make daily leukocyte counts



in Monkeys 71 and 73; and as controls we counted two fresh monkeys, 74 and 75, which latter received intravenously 10 c.c. defibrinated blood drawn from a case of mumps at that time in the hospital. Our counts are shown in the charts, which give the curves of the leukocyte

counts, all of which were made about the same time in the afternoon. The animals were fed at the same hour in the morning. According to Klineberger and Carl,³ the leukocyte count in apparently healthy monkeys may vary as much as 500 per cent. Indeed, their series of



counts show variations from 3,950 to 31,500. They give the average count as 7,470. They used several species of monkeys in making these counts.

Finally, in addition, various quantities of blood from all cases were planted in freshly boiled glucose broth fermentation tubes. Some

3. Die Blutmorphologie Laboratoriums-Tiere, Leipzig, 1912, p. 62.

of these were kept at room temperature and some at 37 C. for several days. Growth appeared in several of the tubes, even after most careful technic, but probably all of the growths might be safely considered as contaminations.

From our work we feel justified in concluding that if dengue is conveyable to the rhesus monkey by intravenous or subcutaneous inoculation of defibrinated blood, these animals do not show the disease by changes in temperature, appearance of skin eruption or any of the ordinary symptoms of illness; further that it is doubtful whether they show any definite and characteristic change in the white blood-cell count, but the results obtained by us are perhaps sufficiently suggestive to invite further effort.

THE BACILLUS ABORTIVUS EQUINUS AS AN ETIOLOGICAL FACTOR IN INFECTIOUS ARTHRITIS OF COLTS *

EDWIN S. GOOD AND WALLACE V. SMITH

(From the Division of Animal Husbandry, Kentucky Agricultural Experiment Station, Lexington, Ky.)

The disease in question is known by a number of names, such as "joint evil," "joint ill," "rheumatism," "pyosepticemia of sucklings," "pasteurellosis neonatorum," "navel ill," "omphalophlebitis septica," "pyemic and septic joint disease of sucklings" and "septic arthritis."

Septic arthritis is an infectious disease of new-born animals, found most commonly in young colts. The disease is quite common in this and foreign countries and spreads rapidly in large stables, abounding in soiled bedding and where aseptic precautions are unheeded. In this territory, infectious arthritis is often found associated with colts that come alive in a stud of mares afflicted with infectious abortion.

It is claimed that infectious arthritis is due to an infection starting in the unhealed umbilicus at the time of or soon after birth, and by multiplication the micro-organisms causing the trouble reach the blood-stream, finding an especially suitable soil for their growth in the lungs, liver and on the synovial membranes and fluids of certain joints. Death ensues from the invasion of micro-organisms producing a general septicemia, or from inflammatory process of different internal organs and membranes of the joints of the body.

Foreign investigators have isolated a bacillus belonging to the hemorrhagic septicemia group, as well as a virulent strain of the colon bacillus.¹ Investigators in this country usually attribute the disease to a streptococcic invasion.²

Our investigations relative to the etiology of this disease among colts have been rather limited. In 1911, Dr. R. M. Bryan of Lexington sent to this laboratory some material which he had obtained by draining a large abscess formed at the hock of a young colt. A bacteriological examination revealed a streptococcus in large numbers.

* Received for publication, June 27, 1914.

1. Hutyrá and Marek: Pathology and Therapeutics of Diseases of Domestic Animals, 1912, 1, p. 159.

2. Moore: The Pathology of Infectious Diseases of Animals, 1906, p. 25.

The second subject that we secured was a well-developed colt which had died from infectious arthritis. The hock, knee and ankle-joints were very much swollen and contained much pus; in fact, all joints of the body which we examined contained some pus. In plating this material we were surprised that a number of plates containing streak dilutions showed no growth. In only two or three plates streaked with pus from the joints were a few colonies obtained, but none proved to have been produced by pyogenic organisms. The few scattering colonies referred to above proved to have been produced by a bacillus resembling somewhat the bacillus abortivus equinus. This organism, however, died out so soon on the media used that we did not have the opportunity to make an extended study of it.

The third case was a colt dropped from a mare in which abortion had been produced experimentally the previous year by an intravenous injection of a small amount of the bacillus abortivus equinus.³ This mare was gotten safely in foal at the third service after aborting, and delivered a strong, healthy colt on April 27, 1914. We do not believe that this mare carried over any infection, due to the fact that the colt was very strong when dropped, also to the fact that during the four months previous to giving birth to the colt the blood-serum from this mare did not agglutinate the bacillus abortivus equinus, nor did it respond to the complement-fixation test. This colt was first seen to be ailing on May 13, 1913. The colt did not improve and showed signs of suffering from "joint ill." Dr. J. T. Shannon, who has had much experience with infectious arthritis in colts in this region, saw the animal and pronounced the symptoms typical of that disease. The colt was very much depressed and lay down most of the time. When helped to its feet it walked with difficulty and the movements of the legs produced apparent pain. It seldom nursed unaided. If permitted, it would drink large amounts of water. The pulse was accelerated and the temperature was high, registering 103.5 F. A persistent diarrhea soon set in. The eyes became involved, resulting in the loss of sight of one eye and the partial impairment of the sight of the other eye. At first, there was quite a noticeable swelling of the hocks, which later subsided to a considerable extent. There was, however, no improvement in the gait of the colt as the swelling subsided. The skin was covered with eruptions measuring about 1 cm. in diameter. On May 19 blood was drawn from the jugular vein; the serum did not give the agglutination and complement-fixation tests with antigen made from the bacillus abortivus equinus. The colt died during the night of May 20. On autopsy large areas of the lungs were found to be hepatized. The lymphatic system was congested. The spleen was enlarged and covered with many petechiae. The heart appeared normal. The kidneys were enlarged and somewhat friable. The outer covering of the small intestines contained many blood-spots, ranging in size from a pinhead to 1 cm. in diameter, as is sometimes noted on the small intestines of a hog suffering from cholera. Areas of the heart, lungs, spleen, liver and of the kidneys were seared, and streak dilutions were made from the blood of these organs as per the usual technic. After twenty-four hours' incubation, these streak dilutions showed a growth resembling somewhat that of the bacillus abortivus equinus. That the colon bacillus was also present was evidenced by the odor. After making several plate dilutions, a bacillus having the morphological and the cultural characteristics of the bacillus abortivus equinus was obtained in pure culture. The skin from the hocks, knees and ankles was removed, the places laid bare were washed with a 5 per cent. solution of carbolic acid and the joints were

opened with sterile knives. The synovial fluid of these joints was slightly increased in amount and of a slightly darker amber color than usual—not so much, however, as to denote marked pathological changes. The results of plate cultures made from the fluid contents of these joints showed that a bacillus responding to the morphological and fermentation tests of the bacillus abortivus equinus was present in abundance in this fluid and was not associated with any other organism. An agglutination fluid was made by washing the growth from agar slants inoculated with material from the colt's knee. Bacillus abortivus equinus immune sheep-serum, which completely agglutinated our standard fluid in a dilution of 1 to 1,250, completely agglutinated the fluid made from the organism derived from the colt in a dilution of 1 to 1,250. Bacillus abortivus equinus immune horse-serum, which completely agglutinated our standard fluid in a dilution of 1 to 1,000, completely agglutinated the fluid noted above in a dilution of 1 to 1,250. By using .01, .02, .03, .04, .05, .06, .07 and .08 c.c. of an antigen made from slants streaked with the organism obtained from the colt, with the bacillus abortivus equinus immune sheep-serum whose titer was .02, and with the bacillus abortivus equinus immune horse-serum whose titer was .02, the complement was completely fixed, with no absorption in tubes containing five times the amounts of antigen in the first four tubes above.

From all the above tests, we feel sure that the organism isolated from this colt suffering from infectious arthritis is the same as the organism causing infectious abortion in mares. We are of the opinion that the colt was infected by way of the umbilicus. That the blood-serum of this colt did not respond to the agglutination and complement-fixation tests, as is noted of mares afflicted with infectious abortion, was due, no doubt, to the shortness of the infection, thus not allowing sufficient time for the development of immune bodies. Our observations show that immune bodies in mares do not develop for from ten to fourteen days after subcutaneous and intravenous injections of the bacillus abortivus equinus. A mare had died the year before, in the stall in which this colt was dropped, from a chronic diarrhea due to a heavy feed of this bacillus. The stall was cleaned and thoroughly sprayed with a coal-tar disinfectant. However, as this stall has a dirt floor, it is evident that disinfection would be difficult.

At the present time it is impossible to say as to what rôle the bacillus abortivus equinus plays in the disease of infectious arthritis of colts, but that it is capable of producing this disease, as are the other germs noted by investigators, is evidenced in this instance.

AN ANAEROBIC VIBRIO ISOLATED FROM A CASE OF ACUTE BRONCHITIS *

RUTH TUNNICLIFF

(From the Memorial Institute for Infectious Diseases, Chicago.)

An organism resembling the cholera vibrio was found in fair numbers in the sputum of a patient suffering with acute bronchitis.

It is a strict anaerobe. It was isolated in pure culture after three days' incubation at 35 C. from a shining clear colony about 1 mm. in diameter, near the fluid of condensation of a tube of goat blood-agar. In subcultures, after two or three days' incubation, pin-point colonies appear on the surface of blood-agar. The growth is more glistening



Fig. 1.—Pure culture, 4 days old. Carbol-gentian-violet. $\times 1200$.

on alkaline blood-agar than on plain agar. A profuse growth occurred on the surface and in the fluid of condensation of one tube of Löffler's blood-serum. No change was produced on the surface, but the fluid of condensation became clouded. There was a faint growth in the line of inoculation in a stab of ascites tissue agar and on ascites agar, but none in milk, plain or ascites broth or on plain agar. There was no odor from the cultures. The vibrio was grown with great difficulty, successful cultures being obtained in only a few of the tubes inoculated.

* Received for publication, June 25, 1914.

The vibrios measure from 2 to 4 microns in length and one-fourth micron in width. They show generally one or two curves, but sometimes more. Straight forms are also seen. Chains of two or more vibrios are occasionally observed. The ends are generally pointed and not parallel to the long axis. A ring may be seen at times, attached to one end. Rather long thick filaments are sometimes observed. When stained by the Zettnow method, the vibrio shows one long fine wavy flagellum attached to its extremity.

The vibrio is gram-negative. It stains fairly deeply with methylene-blue, but not so intensely as with carbol-gentian violet or carbol-fuchsin. It is colored blue with Giemsa's stain. Irregular staining is frequently observed.

With dark-field illumination the vibrio is seen to be flexible and commonly very motile, not progressively, but moving around its own axis, often appearing to have a corkscrew motion. The vibrio died before its pathogenicity was tested.

This vibrio differs from *Spirillum sputigenum* isolated by Mühlens,¹ in the number and arrangement of the flagella and the staining reaction with Giemsa, *S. sputigenum* generally possessing two or three flagella, one usually attached to its concave side and staining red with Giemsa. It differs from *Spirillum crassum* and *Vibrio tenuis* of Veillon and Repac² culturally, and in not producing any odor.

1. *Centralbl. f. Bact.*, I, Orig., 1909, 48, p. 523.

2. *Ann. Past. Inst.*, 1912, 25, p. 300.

A PLEOMORPHIC BRANCHING ORGANISM ISOLATED FROM A CASE OF CHRONIC RHINITIS *

RUTH TUNNICLIFF

(From The Memorial Institute for Infectious Diseases, Chicago.)

A pleomorphic branching organism was isolated in pure culture twice from a case of ordinary chronic rhinitis, in a woman 25 years old.

It is an anaerobe, but occasionally a slight growth occurs aerobically. The colonies on goat blood-agar appear after six days' incubation at 35 C. as scarcely visible, clear, pin-point colonies. In dextrose agar stab cultures there is a faint hazy growth. No multiplication



Fig. 1.—Pure culture, 4 days old. Carbol-gentian-violet. $\times 1200$.

occurs on plain, dextrose, alkaline or horse-serum agar slants or stabs, except dextrose, none on potato, in glycerin or in dextrose, ascites or plain broth with or without tissue. There was no odor from the cultures.

This organism measures one-half micron in width and from 2 to 30 or more microns in length, the average length being about 10. Morphologically, it is very pleomorphic, showing short and long, straight,

* Received for publication, June 25, 1914.

slightly wavy and closely twisted forms. True branching is observed. Ring forms are sometimes seen at one extremity. The ends are generally rounded. Deeply staining bodies are found free, and also inside and attached to the ends and sides of the organism like buds. In old cultures the organism stains irregularly and appears larger than in young cultures. Long unstained spaces sometimes divide the filaments into several parts. Masses of wavy threads like a mycelium are seen in the fluid of condensation of blood-agar cultures.

The organism is gram-negative and not acid-fast. It stains well with methylene-blue, carbol-gentian-violet and carbol-fuchsin. It stains pink with Giemsa, as do the buds and free bodies.

With dark-field illumination some of the short forms are seen to be flexible and appear to move, although no flagella could be demonstrated by the Zettnow method. The long, wavy forms maintain their spiral form and are not motile.

The organism is not pathogenic when injected intraperitoneally into guinea-pigs.

This spiral branching organism is of interest because of the minuteness of its colonies and the evident ease with which it might be an undiscovered contamination, the hazy growth in solid media similar to that of many spirochetes, its general lack of motility and its staining pink with Giemsa.

THE ESTERASE ACTIVITY OF PLAIN AND DEXTROSE BROTH CULTURES OF THE TYPHOID BACILLUS

STUDIES IN BACTERIAL METABOLISM, XXXIX *

A. I. KENDALL AND J. P. SIMONDS

(From the Departments of Bacteriology and Pathology, Northwestern University Medical School, Chicago.)

A series of experiments to be reported soon¹ have shown that both the filtrates of broth cultures of acid-fast bacilli and the organisms themselves contain active lipase. The period of maximum lipase activity of these organisms, furthermore, coincides rather closely in time with the period of maximum vegetative activity of the bacteria. This phenomenon was established by comparing the fat- and ester-splitting power of the filtrates of these cultures with their proteolytic activity under the same conditions of incubation.

Bacteria which are not acid-fast also appear able to split fats. According to Rubner,² this phenomenon is usually a "fat fermentation," not brought about by a fat-splitting ferment (lipase), but rather by the direct action of the bacterial cytoplasm. Other observers, notably Sommaruga,³ and Wells and Corper,⁴ believe a true lipolysis takes place. Several well-known pathogenic bacteria, according to these observers, possess a lipase. It is not definitely determined whether this ferment is a true endo-lipase liberated only when the organisms are disrupted, ordinarily by autolysis, or whether this lipase is, in part at least, a true exo-ferment. The composition of the medium in which these organisms are grown, furthermore, may conceivably influence the nature and extent of the lipolytic activity of these bacteria, and it was with these several possibilities in view that the following experiments were made.

The organism selected was a stock culture of *B. typhosus*, which has been on artificial media for several years. It is perfectly typical in every respect. The media were, respectively, plain nutrient meat-juice-peptone broth, freed from fermentable sugar by the Theobald Smith method, and the same broth to which was added 1 per cent. C. P. dextrose prior to sterilization, which was performed in the autoclave after these media were flaked in 100 c.c.

* Received for publication, June 26, 1914.

1. Next number of this journal.

2. *Arch. f. Hyg.*, 1900, 38, p. 67.

3. *Ztschr. f. Hyg.*, 1890, 18, p. 441.

4. *Jour. Infect. Dis.*, 1912, 9, p. 388.

amounts. The organism (in duplicate) was grown in parallel cultures for several repeated transfers, respectively, in plain and dextrose broth prior to inoculation in these flasks media, to develop whatever latent lipolytic possibilities these two media might induce in the organism. The media, after inoculation with these two strains, were incubated at 37 C. and a flask of each broth was examined at stated intervals. The technic of examination was as follows: The cultures were filtered through sterile Berkefeld filters and the filtrates (with suitable media and ester controls) tested for lipolytic activity according to the procedure described in detail elsewhere.⁵ The bacteria which were held back by the filters were scraped off into toluol water and also tested for lipolytic activity by the same procedure. But 1 c.c. of the filtrate was tested for its activity, representing theoretically but 1/100 of the total amount in the culture, while the entire amount of bacteria from the same culture, so far as they could be recovered, were employed for the same end. The results are given in Table 1.

TABLE 1.

B. typhosus	Days Incubation	Plain Broth					Dextrose Broth				
		Reaction Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. media	NH ₃ Total Nitrogen Per Cent.	Ethyl butyrate		Reaction Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. media	NH ₃ Total Nitrogen Per Cent.	Ethyl butyrate	
					Filtrate c.c. N/50 NaOH	Bacteria c.c. N/50 NaOH				Filtrate c.c. N/50 NaOH	Bacteria c.c. N/50 NaOH
A	1	-0.30	1.4	0.44	0.20	0.05	+2.20	0.70	0.22	0.25	0.25
B	1	-0.30	2.1	0.65	0.25	0.05	+2.30	0.70	0.22	0.25	...
A	3	-0.40	2.8	0.87	0.20	0.05	+2.80	0.70	0.22	0.30	...
B	3	-0.30	2.8	0.87	0.20	0.05	+2.80	0.70	0.22	0.35	0.30
A	5	-0.40	3.5	1.09	0.30	0.05	+3.10	0.70	0.22	0.40	0.25
B	5	-0.50	2.8	0.87	0.35	0.10	+3.20	0.70	0.22	0.50	0.25
A	10	-0.70	3.5	1.09	0.40	0.05	+3.30	0.70	0.22	0.35	0.25
B	10	-0.60	4.2	1.31	0.50	0.05	+3.20	0.70	0.22	0.40	0.30
A	16	-0.80	4.2	1.31	0.40	0.10	+3.70	1.40	0.44	0.40	0.20
B	16	-0.70	4.2	1.31	0.45	0.15	+3.80	2.10	0.67	0.40	0.25

In Table 1, A and B are parallel determinations in duplicate flasks of media. The change in reaction to phenolphthalein is expressed as cubic centimeters acid (+) or alkali (—) per 100 c.c. media, and the increase in ammonia content in milligrams per 100 c.c. media. The percentage of ammonia nitrogen (increase) to the total nitrogen of the uninoculated controls is given, and also the cubic centimeters of N/50 NaOH required to neutralize the acid liberated from ethyl butyrate in twenty-four hours at 37 C. by 1 c.c. of the filtered culture, and all the bacteria separated from the culture, respectively. The media and the ester gave uniformly negative results.

The theoretical total esterase activity is theoretically 100 times that expressed in the table, for but 1 c.c. (1/100) of the total filtrate was used. On the other hand, all the bacteria separated from the filtrate were examined for esterase activity.

5. To appear in *Jour. Infect. Dis.*, 1914.

The growth in dextrose broth was uniformly greater than the corresponding plain (dextrose-free) broth of the same date, hence the difference in esterase activity between the plain and dextrose filtrates shown in the chart is, in part at least, due to this factor.

The bacteria, killed with toluol, recovered by scraping the Berkefeld filter, showed but little esterase activity.

A second experiment was made to determine the esterase activity of larger numbers of typhoid bacilli than could be recovered from the broth cultures. For this purpose six dextrose and six plain agar slants, each of 1.5 by 5 cm. surface, approximately, were inoculated with the "plain" and "dextrose" strains, respectively, and incubated forty-eight hours at 37 C. The bacterial growth was washed off into toluol water and tested in the same manner.

The "plain-agar" culture required 2.40 c.c. N/50 NaOH to neutralize the acid liberated from the ethyl butyrate at the end of forty-eight hours' incubation, while the "dextrose-agar" strain required 2.60 c.c. N/50 NaOH to effect neutralization.

SUMMARY

The sterile filtrates of plain and dextrose broth cultures of typhoid bacilli liberate acid from ethyl butyrate.

The liberation of this acid is due to the esterase activity of a ferment present in these filtrates.

The esterase activity of the filtrates appears to be greater than that of the bacteria separated from the filtrates.

THE BACTERIOLOGICAL AND CHEMICAL EVIDENCE OF THE OCCURRENCE OF A HEXOSE SUGAR IN NORMAL MILK*

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One of the noteworthy cultural reactions of the organisms of the typhoid-paratyphoid-dysentery group is the production of a lilac color, either transient or permanent, in litmus milk. Theobald Smith¹ appears to have been the first to have called attention to this phenomenon. He pointed out that many organisms which ferment dextrose but not lactose produce this color reaction, and that various cultures of these bacteria made from the same milk exhibit the same degree of color change, practically never more nor less for one variety of organism than another, suggesting strongly that each variety is acting on the same substance in the milk, probably a second carbohydrate.

The purpose of this investigation was to determine by bacteriological and chemical examination the substance or substances in milk to which this reaction is attributable.

BACTERIOLOGICAL EVIDENCE

Decomposition Products of the Various Milk Constituents as Possible Sources of Acid Formation.—Milk consists essentially of proteins, fats, salts and carbohydrates, dissolved in water or in colloidal solution.

Proteins may be tentatively ruled out as a source of this reaction by the fact that the products of microbic action on them are usually basic rather than acid in character. A number of organisms of which *B. alkaligenes* is an example, which do not ferment carbohydrates, produce a progressively alkaline reaction in this medium as a result of their action on the milk proteins.

While fats cannot be ruled out by *a priori* considerations, nevertheless the organisms mentioned above do not appear to decompose fats as readily as they decompose proteins or utilizable carbohydrates; moreover, the color-change in litmus milk cultures of typhoid bacilli,

* Received for publication, June 26, 1914.

1. *Jour. Boston Soc. Med. Sc.*, 1897, 2, p. 236.

for example, is found to be the same, whether it is observed in skimmed milk (0.2 per cent. fat) or whole milk (3.5 per cent. fat). If the reaction in this specific instance depended on the utilization of fats, the difference in the fat content of the milk in the instance cited should cause a noticeable difference in the intensity of the color-change.

The salts of milk appear to take no prominent part in their reaction, since an increase in hydrogen ion can occur only by removal of the metal ions from solution; and inasmuch as this color-change may be observed also in dextrose (0.1 per cent.) broth cultures whose salt content is known, an explanation of the phenomenon on the basis of the removal of metal ions of the salts in broth is obviously ruled out.

The tentative exclusion of three of the possible sources of this acid formation focuses attention on the carbohydrate content of milk. With respect to the carbohydrates in milk there is diversity of opinion: some observers claim that lactose alone is found normally in it; others admit that other carbohydrates may occur as normal constituents. Most investigators, however, are non-committal on this point.

The regularity with which this faint acidity is produced in litmus milk by those organisms which are known to ferment dextrose but not lactose would suggest, as pointed out by Theobald Smith, that the color-change is due to small amounts of acid produced by the fermentation of some hexose present normally in milk in small amounts. If this hexose is derived from lactose by hydrolysis, some plausible explanation for the limitation of the cleavage of lactose must be produced. On the other hand, if the color change is due to the fermentation of a portion of the lactose, then one must explain why these organisms form so little acid in the presence of so much lactose; their failure to use more of the lactose cannot be due to any inhibiting action of the acid, since these organisms are able to increase the acid reaction of the culture to the point of precipitating the casein, if dextrose be added to the litmus milk in amounts of from 0.5 to 1 per cent.

It has been shown by Kendall and others² that utilizable carbohydrates exhibit a sparing action for proteins; in other words, when these two food elements are present in a culture the organisms, if able to utilize the carbohydrate at all, will use the latter in preference to the protein, at least for their energy requirement. The sequence of color changes observed in litmus-milk cultures of these organisms is readily

2. *Jour. Med. Research*, 1911, 25, p. 117; *Jour. Biol. Chem.*, 1912, 11, p. 13; *Jour. Am. Chem. Soc.*, 1913, 30, p. 4.

explainable on the basis of this protein-sparing action of utilizable carbohydrates. If some dextrose is present, the organisms mentioned above do not ferment lactose. For example, *B. paratyphosus beta*, *B. enteritidis*, the bacillus of hog cholera, and the Flexner and Shiga types of the dysentery bacillus, as stated previously, produce this lilac color in litmus milk, but after two to eight days, depending on the organism used, the cultures change from this lilac color, "initial acidity," to a blue color, "permanent alkalinity." This phenomenon is explained readily by assuming that a small amount of hexose present at the start is soon exhausted, and the organisms are then forced to act on the proteins for their energy requirement. In the course of the protein decomposition basic substances are formed and the alkaline reaction follows. On the other hand, *B. typhosus* and *B. paratyphosus alpha*, which also produce this initial lilac color change, do not eventually exhibit an alkaline reaction, that is, being dextrose-fermenting organisms, they ferment the hexose of the milk, but their subsequent action on the proteins of the milk does not result in the formation of appreciable amounts of alkaline products. They can, however, give first an acid and then an alkaline reaction in litmus milk if sugar-free beef broth be added to it, or, as shown by Theobald Smith, if 0.1 per cent. dextrose bouillon colored with litmus be used instead of litmus milk. Theobald Smith suggests that this is probably due to the fact that these organisms attack the proteins of the beef broth readily, but do not decompose milk proteins readily. If these organisms act at all on milk proteins, the products are not appreciably alkaline, as is shown by the permanency of the acid reaction which appears during the first twenty-four hours of incubation in litmus milk.

POSSIBLE ORIGIN OF A HEXOSE SUGAR IN STERILE MILK

There are at least two explanations which might account for the presence of hexose in milk. Either it is present as (a) dextrose and galactose, the products of hydrolytic cleavage of lactose, produced by the high temperature and pressure incidental to the process of sterilization in the autoclave, or as (b) dextrose which has passed from the circulating blood of the cow into the milk during the normal process of lactation.³

(a) With regard to the first possibility, it should be noted that litmus milk is prepared by adding litmus solution to fresh milk and

3. Seegan: *Pflüger's Arch.*, 1887, 50, p. 48; Miura: *Ztschr. f. Biol.*, 1895, 32, p. 279.

sterilizing it at 120° C. for fifteen minutes. Lactose is said to be hydrolyzed readily at high temperatures, its hydrolytic products being dextrose and galactose. This hydrolysis would explain the presence of traces of hexose sugar in milk, if it occurred regularly during the process of sterilization.

(b) On the other hand, a consideration of the anatomy of the mammary gland suggests that this sugar may be simply dextrose, and that it may be secreted as a normal constituent of milk. The mammary gland is richly supplied with blood-vessels and lymphatics which lie in intimate contact with the cells actively engaged in the production of milk. During lactation the secretory cells increase in length, extending toward the lumen of the gland tubules; finally, the much-distended cells rupture, and the fluid contained in them escapes at the lumen end of the gland into the alveolus. It is fair to assume, therefore, that in a site of such physiological activity, involving a rich blood-supply, some of the dextrose of the blood might appear regularly in the secretion. Various other body fluids, such as normal urine (Baisch⁴), the transudates and exudates, and even the vitreous humor (Pantz⁵) have been shown to contain dextrose (although in much smaller amounts), and its presence is explained by a similar assumption, that is, that this dextrose comes from the circulating blood of the animal.

BACTERIOLOGICAL TESTS TO DETERMINE THE ORIGIN OF THE HEXOSE SUGAR IN STERILE MILK

The following experiments were made to determine if the lactose of neutral milk is hydrolyzed to dextrose and galactose when heated in the autoclave for fifteen minutes at a temperature of 120° C.

One hundred c.c. of milk were inoculated with *B. paratyphosus alpha* and incubated forty-eight hours. This culture, when it was colored with litmus, showed the lilac reaction characteristic of cultures of organisms fermenting sugars with six carbon atoms. This lilac-colored sample was cautiously neutralized with N/10 NaOH until the color matched that of a sterile litmus milk sample used as a color control.

In the tests requiring color comparisons proportionate amounts of milk and litmus were always used, since cultures containing different proportions of litmus show varying intensities of color changes. One may obtain milk free from the fermentable substance, but identical in color with sterile unchanged litmus milk, by this procedure.

The sample was now divided into two parts, D+ and D°, and 0.05 per cent. dextrose added to part D+. Both portions were then autoclaved at 120° C.

4. *Ztschr. f. Physiol. Chem.*, 1895, 20, p. 249; also Lemaire: *Ibid.*, 1895, 21, p. 442.

5. *Ztschr. f. Biol.*, 1895, 32, p. 236.

for fifteen minutes. It is evident that if autoclaving causes appreciable hydrolysis of lactose in neutral milk, both D+ and D° will now contain hexose sugars, dextrose and galactose, whereas if hydrolysis has not taken place only D+ will contain a hexose sugar, that is, dextrose, which, it will be remembered, was added to it before reautoclaving. To determine if this was the case, D° was reinoculated with *B. paratyphosus alpha*, incubated forty-eight hours, and then compared colorimetrically with D+, the uninoculated sample. No detachable change in color had taken place, showing that the high temperature and pressure incidental to the process of autoclaving did not increase the fermentable sugar content. If hydrolysis had occurred, a slight acidity would have been produced in D° caused by the acid of fermentation of the hexoses, dextrose and galactose.

To show that litmus milk subjected to such treatment can still give the reaction, part D+, to which had been added 0.05 per cent. dextrose, was now inoculated with a loopful of the milk from D°, which, it will be remembered, did not show the color change after forty-eight hours' incubation. After incubating D+ for forty-eight hours, the lilac color change had appeared, when it was compared with D°, which remained neutral. The negative result in D°, therefore, is best explained by the absence of a fermentable substance. The temperature incidental to the process of sterilization, therefore, cannot be responsible for the presence of this fermentable substance in litmus milk in this instance.

A quantitative estimation of hexose in milk was made by comparing the degree of lilac color change produced by the fermentation of varying amounts of dextrose in litmus milk, and by titration of the acid produced by fermentation.

In this test it was necessary to use samples of milk from which the fermentable substance had previously been removed through fermentation, and then neutralized to the color of a control sample of sterile neutral litmus milk. These samples were prepared by the method used in preparing the sample D° just described. After they were so prepared, varying amounts of dextrose (0.05, 0.06, up to 0.15 per cent.) were then added to the sample, respectively, and after sterilization each sample was inoculated with *B. paratyphosus alpha*. These samples containing known quantities of dextrose were then incubated twenty-four hours, and it was found that the one containing 0.08 per cent. of dextrose corresponded most closely in color with that observed in normal milk cultures. This method of comparing the degree of color-change, therefore, shows roughly 0.08 per cent. dextrose in milk.

In the method of titrating the acid formed by fermentation a twenty-four-hour normal milk culture and the twenty-four-hour 0.08 per cent. dextrose culture described above were titrated to neutrality, and found to require the same amount of alkali, namely, 9.2 c.c. of 10/n NaOH. This amount of alkali corresponds to 0.083 gm. of lactic acid and to 0.083 gm. of dextrose, according to the equation $C_6H_{12}O_6 = 2 H C_3H_5O_3$. It cannot be stated positively that this acid is lactic acid, since there is no specific test for this substance which is applicable under these conditions. It can, however, be confidently stated that formic acid is not present in appreciable quantity.

A liter of normal milk was inoculated with *B. paratyphosus alpha* and incubated seventy-two hours. The culture was then concentrated to a volume of

100 c.c. by evaporation and then extracted with ether. The ether extract gave a definite Ufflemann test for lactic acid. This test, however, as stated above, is not conclusive under these conditions. A second liter sample was similarly prepared and incubated. The culture was shaken up with freshly precipitated cupric oxid. After evaporating to 50 c.c. and then acidifying with sulphuric acid in order to convert the copper formate into formic acid, a few cubic centimeters of this sample were distilled and the distillate tested with ammoniacal silver nitrate. No reduction appeared. A control test similarly carried out, using *B. coli*, however, gave a positive test.

These two methods, therefore, agree in showing an amount of fermentable substance in milk corresponding to about 0.08 gm. of dextrose. In other words, in (1) the intensity of the color change in normal litmus milk cultures corresponded to the color change produced by the fermentation of 0.08 gm. of dextrose in 100 c.c. of neutral milk; in (2) the amount of N/10 NaOH required to neutralize the acid formed in a normal litmus milk culture corresponded to 0.083 gm. of dextrose (estimated as lactic acid) in 100 gm. of milk.

Experiments were made to determine if this lilac-color reaction can be obtained in milk which has not been subjected to the high temperature used in the sterilizing process, that is, in milk directly from the cow and untreated in any manner.

Milk that is to be used for cultural purposes must be sterilized; the usual method is to autoclave it at 120 C. for fifteen minutes. The evidence thus far presented for the occurrence of a hexose sugar in milk is based on bacteriological reactions obtained with milk which has been prepared in this manner. It is desirable to make similar tests, substituting absolutely freshly drawn milk for that which has been subjected to a variety of manipulations. If the reactions are as definite in the unchanged milk, they would lend additional evidence to the view that the fermentable substance is not produced in the process of autoclaving—in other words, that it is a normal constituent of milk as it is drawn from the cow. It is necessary to obtain milk samples free from bacterial contamination for this purpose. Bacteria are normally present in the udder, especially in the teats and in the upper parts of the milk ducts. Harding and Wilson⁶ have shown, however, that the middle portion of the milking, especially of the front quarters of the udder, shows a very low bacterial count. The milk samples employed in this test were obtained by discarding the fore milk of the right front quarter of the udder and drawing the middle portion through a sterile glass cannula, one end of which was inserted into the teat, the other end passing through the cotton plug of a sterile 250 c.c. flask. This milk was then removed into sterile test tubes from the flask by a sterile pipet in 10 c.c. quantities. Half of the samples thus obtained were then inoculated with *B. paratyphosus alpha* and the entire number of samples placed in the incubator at 37 C. After twenty-five hours all the samples, both inoculated and uninoculated, were colored with litmus. The difference in color of the inoculated and uninoculated tubes was unmistakable; the inoculated samples showed uniformly the typical

6. *Tech. Bull.*, 1913, No. 27, N. Y. Agricultural Experiment Station.

lilac-color change mentioned above, while the uninoculated samples were uniformly neutral. This shows conclusively that the reaction may be obtained in freshly drawn milk.

CHEMICAL EVIDENCE

The literature relating to the chemical composition of milk makes no definite mention of the occurrence of a hexose as a normal constituent of milk, yet the constant production of acid as shown by the bacteriological tests given above seems to warrant the conclusion that this lilac-color reaction is due to the formation of acid by fermentation of a hexose sugar. However, it was considered essential to employ some chemical test for verifying the bacteriological reactions.

In a chemical examination of milk for the presence of this substance, the most obvious difficulty was to obtain the whey of the milk free from proteins and other substances which would obscure the subsequent chemical tests, without at the same time causing hydrolysis of lactose into dextrose and galactose in the process.

Another difficulty is encountered in attempting to demonstrate the presence of a trace of a sugar with six carbon atoms in the presence of a relatively large quantity of a sugar with twelve carbon atoms.

Polariscopic methods are not sufficiently delicate for this purpose, and of the precipitation tests only one merits any consideration. This is Barfoed's test, which has been used frequently for detecting hexoses in the presence of bioses. This test proves to be delicate to one part in about 40,000 under suitable conditions. A modification of this test was finally devised and is here given:

Barfoed's test is made by boiling the material to be tested, a few drops at a time, with 5 c.c. of a solution containing 4.5 gm. of copper acetate and 1.2 c.c. of 50 per cent. acetic acid per 100 c.c. of water. Some observers claim that lactose is slowly hydrolyzed to dextrose and galactose in the presence of this reagent, which obviously would interfere with the demonstration of any hexose already present. It was found, however, that with the modification as here given, hydrolysis manifested itself only after vigorous and continued boiling for a period much longer than was necessary for the entire process.

One hundred c.c. of milk at 37 C. are shaken up with 2 gm. of copper acetate and filtered at once. The resulting clear whey is then titrated with silver nitrate solution to remove the chlorids which interfere with the subsequent precipitation of cuprous oxid in the last step in the process. It should be emphasized that the least excess of silver nitrate is to be avoided, since the lactose as well as any hexose sugar present will cause its reduction, thus obscuring the reaction. On the other hand, if too little silver nitrate is added, the unprecipitated chlorids, even in very small amounts, prevent the precipitation of cuprous oxid as mentioned above. For this reason the filtrate should be tested with silver nitrate, refiltered and again tested until not more than a trace of chlorid can be precipitated from the whey. The chlorid content of different samples of

milk has been found to vary somewhat, and for this reason the removal of the chlorids constitutes the most tedious part of the process. To avoid unnecessary dilution of the whey a 2.5 per cent solution of silver nitrate is used; slightly more than 4 c.c. (usually about 4.3 c.c.) are required for 25 c.c. of the whey.

After filtering out the precipitated chlorids, the whey is then heated to 90 C. and filtered while still hot to remove phosphates. Two parts of this whey when boiled with one part of a solution containing 8 per cent. copper acetate and 1 per cent. glacial acetic acid will show a slight, dark red precipitate of cuprous oxid. The precipitate is so small in amount that the tubes should stand several minutes or even hours; when viewed against a black background by reflected light the precipitate is seen adhering to the sides or settled to the bottom of the tubes. It cannot be seen distinctly by transmitted light.

To determine the ability of various organisms to ferment this reducible substance, the following tests were carried out for each of the following organisms: *B. alkaligenes* and *B. subtilis*—known to be unable to ferment dextrose—and *B. paratyphosus alpha* and *beta*, *B. typhosus*, and the Flexner and Shiga types of dysentery bacilli—known to ferment dextrose. Flasks containing 100 c.c. of sterile milk were inoculated, respectively, with each of the above-named organisms and incubated forty-eight hours. The whey from each flask was then analyzed for reducing substances by the above method, and its absence demonstrated in the cultures of the dextrose-fermenting ones just named. On the contrary, *B. subtilis* and *B. alkaligenes*, non-dextrose-fermenting bacteria, had not appreciably diminished the original quantity of this reducing substance, as shown by the fact that the cuprous oxid precipitate appeared in the usual quantity. In other words, this reducible substance, whatever it may be, is not appreciably reduced in amount by non-dextrose-fermenting organisms, but it is quantitatively removed by those fermenting dextrose. Furthermore, analyses of 100 c.c. samples of milk, after incubating at 37 C. for periods of eighteen, twenty-four, thirty-six and forty-eight hours, showed that *B. typhosus* and *B. paratyphosus alpha* and *beta* could remove this reducible substance completely in twenty-four hours; while the Flexner and Shiga types of the dysentery bacilli required forty-eight hours' incubation to completely remove it. These observations accord with what is known of the relative vegetative activity of these organisms, and therefore lends additional support to the assumption that this reducible substance is a hexose, fermentable by them.

It might be objected at this point that certain substances may have been present in the material introduced at the time of inoculation, or formed during the growth of the dextrose-fermenting organisms, which may have prevented the precipitation of cuprous oxid, thereby making the test appear to show absence of hexose, as happens in the case when all the chlorids are not removed. This possibility of error was avoided by the use of the following control tests: each of the forty-eight-hour-old cultures of the dextrose-fermenting organisms mentioned above were rendered protein-, chlorid- and phosphate-free by the process just outlined, and in each instance like amounts of whey, now presumably free, by fermentation, from the reducing substance, were placed in two test tubes, A and B. The required amount of the reagent was then added to each tube, and to A was added dextrose in a concentration of 0.01 per cent. After boiling both tubes, A and B, a distinct precipitate was obtained in A and none in B. The negative test in Tube B shows that the organisms have reduced the quantity of reducible substance at least below the concentration recognizable by this test, because Tube A, known to contain 0.01 per cent. dextrose, gives a positive test. This control also shows that lactose is not appreciably hydrolyzed either by the

reagent itself or by the process of freeing the wheys of the proteins, phosphates and chlorids, otherwise Tube B, which contained no addition of dextrose, would give a reduction with the dextrose and galactose so formed. Furthermore, the control enables one to determine if chlorids, which might have inadvertently not been removed, are interfering with the test. In such a case, Tube A, known to contain dextrose, would then fail to give the precipitate.

Having now a method for proving the absence of a hexose in milk after incubating with some dextrose-fermenting organism, the next step in the investigation was to verify by chemical methods whether or not some hexose is produced by the hydrolysis of lactose during the normal process of sterilization.

Two hundred c.c. of sterile milk were inoculated with *B. paratyphosus alpha* and incubated forty-eight hours. To determine if this milk now contained the reducible substance, the sample was divided into two equal parts, A and B. A was analyzed by the above method and shown to be free from this reducible substance. The other portion, B, shown by analysis of A to be free from reducible substance, was then autoclaved in the usual manner for sterilizing milk for cultures, that is, at 120 C. for ten minutes. If sterilization is responsible for the formation of dextrose and galactose in milk, chemical analysis of this latter portion should show the usual cuprous oxid precipitate. The analysis, however, failed to show the presence of a reducing substance, indicating that if hydrolysis of lactose had taken place in the process of autoclaving, the amount of sugar with six carbon atoms so produced was insufficient to give the test.

A number of 10 c.c. samples of normal milk were analyzed by the method given above and the cuprous oxid precipitates weighed in each instance. The average weight of precipitate obtained from 10 c.c. of milk was .003 gm. of cuprous oxid. Other 10 c.c. normal milk samples were then inoculated and incubated in order to free the samples of the reducible substance already present. These samples were then treated with 0.1 per cent. dextrose and analyzed by the above method. The average weights of cuprous oxid precipitate from each of these samples was .005 gm. According to the proportion .003 g.: .005 g.: x per cent. : 0.1 per cent., the amount of reducing substance in milk in terms of dextrose should be .06 per cent. It will be recalled that the method of quantitative estimation of fermentable substances by titration of the acid of fermentation showed an amount corresponding to .08 per cent. dextrose. Milk directly from the cow would probably show 0.1 per cent. or more, as shown by Theobald Smith's estimates. Dextrose being the most frequently and easily fermented of all the fermentable sugars, would naturally occur in slightly varying amounts in market milk, depending on its past history and the age of the sample. Occasionally a sample of milk may fail to give the lilac-color change with dextrose-fermenting organisms. This is apparently due to the fact that the hexose has been decomposed by the action of the bacteria in the milk prior to the addition of the litmus.

It was shown by bacteriological tests that milk freshly drawn from the cow and untreated in any manner could give the typical lilac-color change after inoculation, incubation and coloring with litmus, showing that the substance to which the color change is attributable is normally

present in milk. If now it can be shown that this substance reduces a reagent reducible by sugars containing six carbon atoms, all the evidence will be in favor of the view that this substance, fermentable by dextrose-fermenting organisms and reducible by a dextrose-reducing reagent, is probably dextrose which has found its way from the circulating blood of the cow.

In this test the milk was drawn from the cow directly into flasks and divided into 100 c.c. quantities. The entire process of the analysis was carried out immediately after milking. On boiling the wheys of these samples with the reagent as before, the usual dark-red cuprous oxid precipitate, apparently in the usual quantity, was obtained, showing that regardless of where and by what mechanism this substance had its origin, a second reducible, fermentable carbohydrate, probably dextrose, occurs as one of the normal constituents of the mammary secretion, in amounts approximating the concentration of dextrose found in the circulating blood of the cow.

CONCLUSION

Bacteriological and chemical evidence has been presented which indicates that milk normally contains a substance which reacts like dextrose.

THE TREATMENT OF TETANUS BY ANTITETANIC SERUM *

AN ANALYSIS OF 225 CASES, 1907-1913

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The mortality of tetanus treated symptomatically is estimated by most observers at from 78 to 90 percent. A few authors have given figures considerably lower than this, but these latter vary so greatly from the average that it seems that the apparent reduction in mortality must have been due to some unrecognized factor of selection in the compilation of statistics.

TABLE 1
MORTALITY OF TETANUS WITHOUT SERUM
(Hospital Statistics Collected by Permin)

	Percent		Percent
Larrey	82.5	Fricker	88.8
Poland	86.0	Posselt	80.0
American Civil War	89.3	Faber	82.5
Richter	88.0	Sormanni	44.0
Demme	85.7	Kowalski	34.9
Rose	78.0		

A number of factors combine to influence the prognosis of the individual case.

Tetanus is commonly divided into acute and chronic, according to whether the interval between the injury and first appearance of symptoms is eight to ten days or less, or more than ten days. This arbitrary division affords a convenient method of estimating the probable severity and mortality of the disease in a majority of instances. Another criterion of the severity of tetanus is afforded by the extent and rate of development of the symptoms after the appearance of first stiffness of muscles, but this evidence is obviously not available at the onset of the disease.

In experimental tetanus produced by the injection of known amounts of tetanus toxin, the incubation period, rapidity of development of symptoms, elapsed time till death, and the mortality are direct functions of the size of the dose. The site of injection also is important. In tetanus developing after wounds the problem is

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more complicated because the toxin is not received all at once, but is absorbed gradually as fast as produced. The rate of production depends in turn both on the amount of organisms introduced and on whether they find favorable or unfavorable conditions of growth. Conditions of growth may be favorable at once after the receipt of the wound, as in a deep puncture, or extensive laceration, or multiplication of the organisms may begin only after several days or weeks have elapsed.

In the majority of cases, the incubation period expresses with a fair degree of accuracy the combination of these factors, although not infrequently some one element such as conditions in the wound, extent of surface injured, or amount of infection introduced may

TABLE 2
MORTALITY OF TETANUS IN DENMARK
Cases Treated Without Serum (Permin and Faber)

Incubation	Lived	Died	Mortality, Per Cent
10 days and less	5	89	94.7
Over 10 days	17	40	70.2
Unknown	20	28	58.3
Totals	42	157	78.9

Cases Treated with Serum (Permin)

Incubation	Lived	Died	Mortality, Per Cent
10 days and less	25	67	72.8
Over 10 days	34	23	40.4
Unknown	21	19	47.5
Totals	80	109	57.7

vary from the usual to such an extent that symptoms appear four or five days after an injury, but develop slowly, so that the course of the disease is relatively mild, or, on the other hand, the production of tetanospasmin may be delayed for a number of days, and then proceed rapidly so that a fulminant disease develops after a long incubation period. If toxin is produced slowly in small amounts, time is afforded for the production of antitoxin by the host, and this probably has a part in the spontaneous recovery from the disease.

The evaluation of antitetanic serum in developed tetanus, a disease which presents so many variable factors influencing the prognosis, is a matter of considerable difficulty, and many experienced

clinicians have doubted whether the results obtained from its use have been any better than those from symptomatic treatment. A study of the statistics of mortality of many hospitals shows that too often this skepticism has been well founded. Opposed to this pessimistic view are the reports of a number of small groups of carefully observed cases, such as those of Ashhurst and John in which the evidence seems strong that antitoxin deserved large credit for the recovery of some of the patients.

Permin¹ has collected statistics in Denmark of the mortality of tetanus treated with and without serum and finds a mortality of 78.9 percent in 199 cases treated without serum, as compared with a mortality of 57.7 percent in 189 cases receiving serum.

TABLE 3
CASES TREATED WITH SERUM

Incubation	Total Cases	Died	Recovered	Mortality, Per Cent
5 days and less	38	27	11
6 days	13	15	3
7 days	21	16	5
8 days	17	14	3
9 days	24	17	7
10 days	13	7	6
	131	96	35	73.28
11-15 days	47	22	25	
16 days and over.....	22	6	16	
	69	28	41	40.57
Incubation known	200	124	76	62.00
Incubation unknown	25	15	10	60.00
Total cases	225	139	86	61.77

CASES WITHOUT SERUM				
10 days and less	12	11	1
Over 10 days	4	2	2
Unknown	5	5	0
Total	21	18	3	85.7

The collection of the cases* of tetanus here analyzed was undertaken with the object of ascertaining in the first place what results

1. Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1913, 27, p. 1

* All cases reported by any one observer are included in so far as the completeness of the data permit. The data of 80 cases were obtained by me directly from hospital records. About 20 cases occurred in the private practice of physicians. The remainder were treated in hospitals, for the most part large metropolitan—including Bellevue, Massachusetts General, Boston City, Buffalo General, Philadelphia General, Royal Victoria and Cook County, Wesley, Presbyterian and Michael Reese. To those who have kindly assisted in the collection of cases my best thanks are extended. I am especially indebted to Drs. O. Berghausen, Cincinnati; R. J. Carlisle, New York; Russell S. Fowler, Brooklyn; C. D. Fox, Philadelphia; F. S. Graves, Boston; G. W. Green, Chicago; H. C. Marble; A. C. Morgan, Philadelphia; John McCrae, Montreal; E. R. McGuire, Buffalo; J. J. Toland, Frankford, Pa.; Frank Vander Bogert, Schenectady; H. B. Wadsworth, Washington, Ind., and to others who supplied data of smaller numbers of cases.

are being obtained in this country with antitetanic serum in the treatment of tetanus (during the period 1907 to 1913), and in the second place, whether the failures in some cases may not be ascribed to the faulty and insufficient method of giving the serum. Statistical studies of cases collected from the literature are necessarily subject to error, and particularly in tetanus, such tabulations have led to conclusions favorable to serum treatment to a degree unwarranted by the actual facts.

With one or two possible exceptions, none of the cases in this series have been reported, and all were obtained by personal correspondence from hospital or private records. In all, this collection includes 225 cases treated with antitetanic serum, and 21 cases which received no serum.

Table 3 summarizes the mortality of the several classes of cases. The mortality of all cases receiving serum is 61.77 percent; in 21 cases without serum the mortality is 85.7 percent. The number of cases in this latter group is, of course, too small to establish a mortality for tetanus, but it agrees with the generally accepted figures of many writers on tetanus, 78 to 89 percent. It appears therefore that the mortality of cases in this series is about 20 percent lower in cases receiving serum than in those treated without serum. A further analysis of the cases with respect to the size of the dose, method of giving the serum, and duration of symptoms before the serum was given suggests the reasons for failure in some cases.

DISTRIBUTION OF TOXIN IN TETANUS

In the average patient suffering from tetanus in whom symptoms have just appeared the following conditions are present. Some of the toxin has already reached the central nervous system, and trismus or other evidence of tonic spasm suggests the diagnosis; another portion of toxin is ascending centralward in the nerves supplying the infected extremity; a still larger amount of toxin is present in the blood and is being taken up and is traveling centralward along the other nerves of the body. Some of these patients have already received in the peripheral and central nervous system a fatal dose of toxin when they are first seen, and will die no matter what is done. However, patients apparently hopeless, in whom convulsions have already begun, may recover, and since at the present time there seems

to be no criterion by which we may be sure that the fatal dose has already reached the central nervous system, all cases should receive energetic treatment.

The necessity for haste is evident. The toxin still free in the blood must be neutralized as quickly as possible, and for this purpose the intravenous injection of serum is advisable. Next in rapidity of absorption is the intramuscular route, which should be employed if for any reason the intravenous method cannot be used. Antitoxin given subcutaneously reaches the maximum concentration in the blood only toward the end of the second twenty-four-hour period after

TABLE 4
RESULTS WITH RESPECT TO TIME WHEN SERUM WAS GIVEN AND SIZE OF DOSE IN FIRST
TWENTY-FOUR HOURS OF TREATMENT*

Incubation	Large Doses		Small Doses		Mortality	
	Died	Recovered	Died	Recovered	Large Dose	Small D.
A. Cases receive 10 days and less Over 10 days ..	ing first 41 11	serum with 13 15	in 24 hour 21 6	of first 3 3	symptoms: 75.9 42.3	87.5 66.6
B. Cases receive 10 days and less Over 10 days..	52 ing first 11 2	28 serum in 9 8	27 second 24 6 1	6 hours after 0 0	65.0 first symp 55.0 20.0	81.8 toms: 100.0
C. Cases receive 10 days and less Over 10 days ..	13 ing first 10 7	17 serum over 6 10	7 48 hours 7 1	0 after first 4 5	43.3 symptoms: 62.5 41.1	100.0 63.6 16.6
Grand Totals: 10 days and less Over 10 days ..	17 62 20	16 28 33	8 34 8	9 7 8	51.5 68.8 37.7	47.0 82.9 50.0
	82	61	42	15	57.3	73.7

* In this table a small dose = 3,000 units or less subcutaneously; a large dose = over 3,000 subcutaneously or 3,000 or less intraspinally or intravenously.

injection. This delay in absorption may entail a lapse of time sufficient to allow the development of a fatal intoxication. It is true that in some cases the formation of the toxin may proceed slowly, so that the subcutaneous injection of antitoxin may prove life saving, but in the ordinary acute case a dose of 1,500 or 3,000 units given subcutaneously can be of little immediate value in neutralizing toxin in the blood and staying the course of the disease.

The importance of intraspinal injection of antitoxin as a means of dealing with toxin that has already reached the central nervous system has been recently emphasized by Park, who has shown that

animals in which tetanus has already developed may be saved by antitoxin in adequate doses and that the results are better and smaller doses required when the antitoxin is given intraspinally than when other routes are employed. These experimental results, together with a number of favorable results in human tetanus, indicate that the intraspinal injection of antitoxin should be urged in all cases of tetanus.

The intraneural injection of antitoxin serves to neutralize that portion of toxin peripheral to the point of injection in the nerves supplying the infected extremity, but from what we know of the distribution of toxin in the body in human tetanus, the instances in which this method alone is life saving must be extremely rare.

In Table 4 the cases are divided into groups with respect to (1) the incubation period, (2) the time elapsing from the appearance of symptoms to the beginning of treatment, and (3) the amount and efficiency of method of injection of the antitoxin during the first twenty-four hours of treatment. This classification may be criticised as faulty in a number of particulars, but taking the cases in the aggregate, it offers a basis of comparison as accurate as can be obtained from the data available. A comparison of the mortalities of cases receiving large and small doses of antitoxin shows the importance of adequate dosage and proper method of injection. In acute tetanus a small dose of antitoxin given subcutaneously does very little good. The reduction of aggregate mortality over the mortality of untreated tetanus is apparently due to the favorable results obtained in the cases where larger doses were employed in an efficient manner.

Some comment is necessary on the relative mortalities of cases receiving large doses in the first and second twenty-four-hour periods. The lower mortality in the second group (B) is obviously due not to the postponement of treatment, but to the fact that a large number of cases of acute tetanus die within twenty-four to thirty-six hours after the appearance of symptoms. Such cases will necessarily appear for the most part in the first group (A) and the second group (B) will accordingly show a lower mortality, through this unavoidable element of selection. Likewise the third group (C) contains some cases with slower onset, milder course and correspondingly less grave prognosis, in which necessity for rapid neutralization of toxin is

less urgent, and the difference between the results obtained by large and small doses of antitoxin disappears. It should be remembered, however, that these cases are distinguishable only after the termination of illness, and the fact that they occur should be given no weight in planning the treatment of the individual case. Every case of tetanus is extremely serious.

Perhaps the most serious criticism to be urged against this classification of cases is that the grouping of cases as to duration of symptoms prior to the giving of antitoxin into periods of twenty-four hours, does not take into account whether patients received

TABLE 5
RESULTS WITH RESPECT TO TIME WHEN SERUM WAS GIVEN AND TOTAL AMOUNT*
OF SERUM USED

Incubation	Large Doses		Small Doses		Mortality Percent	
	Died	Recovered	Died	Recovered	Large Dose	Small D.
A. Cases receive 10 days and less Over 10 days ..	ing first 35 10	serum with 14 16	in 24 hours 27 7	of first 2 2	symptoms: 71.4 41.5	93.1 77.7
B. Cases receive 10 days and less Over 10 days ..	45 ing first 11 2	30 serum in 9 8	34 second 24 6 1	4 hours after 0 0	60.0 first 55.0 20.0	89.4 symptoms: 100.0
C. Cases receive 10 days and less Over 10 days ..	13 ing first 6 4	17 serum over 7 9	7 48 hours 11 4	0 after first 3 6	43.3 symptoms: 46.1 30.7	100.0 78.5 40.0
Grand Totals: 10 days and less Over 10 days ..	10 52 16	16 30 33	15 44 12	9 5 8	38.4 63.4 32.6	62.5 89.7 60.0
	68	63	56	13	51.9	81.1

* A small dose = 10,000 units or less subcutaneous. A large dose = over 10,000 subcutaneous or less than 10,000 intraspinal or intravenous.

first treatment at the beginning or the end of the first twenty-four hour period, i. e., whether antitoxin was administered immediately after symptoms appeared or whether twenty-four hours elapsed before treatment was begun. As pointed out by Park, twenty-four hours is a long time in tetanus. The data available in the cases in this series did not allow of a division of time smaller than twenty-four-hour periods. With the more general appreciation of the necessity for treatment immediately after the appearance of symptoms, more accurate data, which should be stated in hours rather than days, will be available for statistical study.

In Table 5 the results of treatment with antitetanic serum are tabulated with the same grouping as in Table 4, but with respect to the total amount of serum used in each case. In this tabulation a small dose is defined as 10,000 units or less, given subcutaneously, and a large dose as more than 10,000 units subcutaneously or less than 10,000 units, if given intravenously or intraspinally. Most of the cases receiving small doses were given 1,500 or 3,000 units per day, subcutaneously, an amount obviously too small to be of service in an acute case; while most of the cases in the class receiving large doses were given considerably more than 10,000 units subcutaneously, or less amounts intraspinally or intravenously. This purely arbitrary division affords a basis for the comparison of the effects of dosage in the series under discussion, but is not intended as a guide for the treatment of the individual case. It should be noted that even in those cases receiving relatively large doses subcutaneously, the distribution of dosage through the first twenty-four-hour period was such that the maximum concentration of antitoxin could not have been attained until well into the following twenty-four hours.

TABLE 6

MORTALITY OF TETANUS TREATED BY INTRASPINAL AND BY INTRAVENOUS INJECTIONS OF ANTITOXIN, WITHOUT REGARD TO THE TIME TREATMENT WAS BEGUN

	Intraspinal			Intravenous		
	Cases	Died	Mortality, Per Cent	Cases	Died	Mortality, Per Cent
Acute	16	13	81.2	11	8
Chronic	17	5	29.4	5	2
Unknown	4	3	4	3
	37	21	56.7	20	13	65.0

The method of grouping is subject to the same criticisms as that of Table 4, and in addition a further error is unavoidably introduced in that those cases which died early in the disease had less opportunity to receive large doses of antitoxin, and consequently increase the percentage of mortality of the cases receiving small doses. This accentuates unduly, the difference between results from large and small doses.

In general the results with large doses are considerably better than those with small doses, although as indicated above, the actual difference is probably not as great as is indicated by the percentages.

RESULTS WITH INTRAVENOUS AND INTRASPINAL INJECTIONS

A limited number of observations on the effects of intravenous and intraspinal methods of injection are available in this series.

Twenty patients, of whom 11 were acute, 5 chronic, and 4 unknown incubation, received intravenous injections of serum either alone or in combination with other methods of injection. The collective mortality of these was 65 percent. Of those who died, however, treatment was begun within eight hours in 1, within twenty-four hours in 5, on the third day in 2 and on the fourth day of symptoms, or later, in 3. In 2 the duration of symptoms before treatment was not noted.

Of 37 patients who received antitetanic serum intraspinally either alone or in combination with other methods of injection, 16 were acute, 17 chronic and in 4 the incubation period was unknown. The collective mortality was 56.7 percent. Of those who died 5 received antitoxin within twenty-four hours after the appearance of symptoms, 5 received first treatment on the second day of symptoms, 4 on the third day, 2 on the fifth day or later, and in 3 the duration of symptoms before treatment was not noted.

The delay of treatment until the second or third day of symptoms, and the small doses (1,500 to 3,000 units) which a number of these patients received, go far toward explaining the failure of these methods to reduce the death-rate in this series below 50 percent. The unfortunate fact that often patients do not apply for treatment until the second or even the third day of symptoms can not be used as an argument against a method of treatment which offers a reasonable hope of success if instituted early in the disease.

MAGNESIUM SULPHATE

Magnesium sulphate was given intraspinally in eighteen cases which also received serum. Four cases, two acute and two chronic, recovered, giving a mortality for the group of 77 percent. In two cases death occurred shortly after the injection, with symptoms of respiratory paralysis.

SUMMARY

From these statistics it appears that the mortality of tetanus treated by tetanus antitoxin is about 20 percent lower than the average mortality of tetanus treated without serum.

The mortality of cases treated by efficient methods and adequate dose is considerably lower than that of cases receiving small doses subcutaneously.

SUGGESTIONS FOR THE TREATMENT OF TETANUS

The prophylactic value of tetanus antitoxin is established, and needs no argument for its support. That tetanus antitoxin properly used may save the life of a patient in whom tetanus has already developed should be more generally recognized, and the treatment employed in every case at the earliest moment possible. Every hour lost before the giving of the antitoxin decreases the chance of saving life. By no means every case will recover but certainly more can be saved than have been in the past five years, and there is every reason to anticipate that with a proper use of antitoxin a mortality considerably lower than the present will be obtained.

It is important that the full effect of the antitoxin should be obtained immediately and this may be accomplished by giving, as outlined by Park, 3,000 to 5,000 units intraspinally and 10,000 to 20,000 units intravenously at the earliest possible moment after symptoms of tetanus appear. On the following day the intraspinal injection may be repeated. The blood remains strongly antitoxic for several days. On the fourth or fifth day 10,000 units should be given subcutaneously to maintain the antitoxin content of the blood.

It is doubtful whether the enormous doses given in some cases over periods of many days are any more effective than the more limited dosage outlined above by which the maximum concentration of antitoxin in the body is attained at once. If only a small amount of antitoxin (3,000 to 5,000 units) is available it should be given intraspinally. Intraspinal and intravenous injections should be given with all the precautions usually employed for these methods.

The danger of immediate anaphylactic shock following intravenous and intraspinal injection of tetanus antitoxin has been suggested as an objection to these methods of giving the serum, but when we consider the relative rarity of fatal instances of such complications in the treatment of diphtheria either by intravenous or subcutaneous injections, and the grave prognosis in untreated tetanus, it is evident that the remote possibility of fatal serum disease should be given little weight in determining treatment.

This use of antitoxin in no respect replaces other necessary recognized non-specific methods of treatment in tetanus. Surgical treat-

ment of the site of infection should be instituted at once. The patient should be placed at rest in bed in a quiet darkened room, and should receive sufficient sedatives to control convulsions, together with adequate supply of fluid nourishment, and attention to the elimination by kidney and bowel. The necessity for large and continued doses of sedatives such as chloral or chlorbutanol should not blind the physician to the possible danger of giving an overdose. The condition of the patient should be carefully watched, and a revision of the standing orders for sedatives made whenever symptoms suggest the decrease or increase of dose.

THE GROWTH AND VIABILITY OF STREPTOCOCCI OF BOVINE AND HUMAN ORIGIN IN MILK AND MILK PRODUCTS *

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While investigations were being made in connection with the epidemics of sore throat in Chicago, in Batavia, Ill., and in Jacksonville, Ill., questions were often asked whether cream, sour milk, buttermilk, ice-cream, butter and cheese were concerned in the transmission of the infecting streptococci. Since these questions have not been carefully studied it was thought worth while to make some observations bearing on them.

So far as we know, all streptococci causing epidemics of septic sore throat have been of the hemolytic variety.† This is certainly true of the more recent epidemics in Chicago, Boston, Baltimore, Jacksonville (Ill.), Concord, N. H., and Wakefield and Stoneham, Mass., in which this particular property was carefully noted. In some of the epidemics, especially the earlier epidemics in England, this property was not investigated, but there is no reason whatever for thinking that any other sort of streptococci was responsible, since the characteristics mentioned in each instance lead one to believe they likewise were of this variety. These statements refer to the organisms isolated directly from the throat, blood, exudates, etc. of persons and also to strains isolated from suspected cows.

While these streptococci are included in the hemolytic group, all are not exactly alike in other particulars. In the reports of the Chicago epidemic, attention was called to the fact that from the blood and exudates, and at times from the throat, certain streptococci were obtained which were highly virulent and which were encapsulated; they produced a clear but distinctly narrower zone of hemolysis than

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† When the term hemolytic property is used in this paper it refers to the appearance of the colonies on human blood agar plates. Those organisms are called hemolytic which cause a distinct clear zone of hemolysis about the colonies when incubated at 37 C. for 24 hours. Many streptococci may produce a narrow greenish, grayish or brownish zone in which, especially after two or more days at times, some clearing of the media may occur. Such strains, however, are not considered true hemolysers and are referred to as non-hemolytic.

the common hemolytic streptococci and they also formed a more spreading and a more mucoid colony on blood agar plates. These observations were confirmed by Stokes and Hachtel¹ in their study of the Baltimore milk epidemic. Now it is possible to cause the ordinary hemolytic streptococcus to take on the properties in question by animal passage. I have already expressed myself on this point,² stating that probably the differences noted may be simply one of virulence; for all of the strains of the moist encapsulated variety have been highly virulent. This is not true of the ordinary strongly hemolyzing strains, some having a low degree of virulence; tho many, it must be admitted, are also highly virulent.

The fact that hemolytic streptococci are concerned in the causation of epidemics of sore throat, centers our attention on this type of streptococci when attempting to trace the source of the infection to milk-supply. On the other hand, there is no reason whatever for attaching importance to streptococci of the non-hemolytic variety so far as a study of the etiology of milk epidemics is concerned. This particular point, it seems to me, should be more generally appreciated, and in all studies on milk streptococci, the hemolytic property should be carefully noted. I call attention to this because not enough emphasis has been placed on this property in the general study of streptococci by many bacteriologists. The work, for example, of Heinemann³ on milk streptococci makes no mention of the hemolytic property and therefore is of little or no value so far as this point is concerned. Likewise, such an excellent recent work as that of Rogers and Dahlberg,⁴ in which a large amount of valuable information about milk streptococci is given, makes no mention whatever of the hemolytic property. Savage,⁵ in his excellent studies on milk, also fails to mention this point when discussing streptococci and their relation to sore throat epidemics. Stowell, Hilliard, and Schlesinger⁶ admit that the hemolytic property is invaluable in the study of pathogenicity, but because it cannot be correlated with other characteristics, they do not consider it in formulating their groups of streptococci.

1. Public Health Reports, 1912, 27, p. 1923.

2. *Jour. Am. Med. Assn.*, 1912, 58, p. 1852.

3. *Jour. Infect. Dis.*, 1906, 3, p. 181.

4. *Jour. Agric. Research*, 1914, 1, p. 491.

5. *Milk and Public Health*, 1912, McMillan & Co., London.

6. *Jour. Infect. Dis.*, 1913, 12, p. 144.

So far as we know at present, any appreciable number of hemolytic streptococci found in cow's milk obtained carefully in sterile tubes directly from the cow's udder always means a diseased condition. Ruediger,⁷ among others, has especially called attention to this point. It is, of course, true that udder disease is often caused by non-hemolytic streptococci, but as already stated, so far as we know, these bear no relation to sore throat. I have examined many of these organisms and they have little or no virulence for animals. On the other hand, the hemolytic streptococci, that I have examined from the udder, are always pathogenic to some degree for animals and usually quite highly virulent. This does not mean that they all are pathogenic for man. We cannot ascertain this point, but the probability is, as pointed out by Savage, that only now and then is this true of such strains.

TABLE 1
GROWTH OF HEMOLYTIC STREPTOCOCCI IN STERILE MILK AT VARYING TEMPERATURES

Temp.	Organism	At Once	3 Hours	6 Hours	20 Hours	48 Hours
10 C.	S. hemolyticus, human....	1,100	1,300	1,400	1,300	1,000
	S. hemolyticus, bovine....	1,000	1,000	900	1,100	900
	S. lacticus.....	1,500	1,500	1,200	1,800	2,000
20 C.	S. hemolyticus, human....	500	400	400	600	1,500
	S. hemolyticus, bovine....	150	20	18	100	500
	S. lacticus.....	40	400	3,000	10,000	many
26 C.	S. hemolyticus, human....	400	400	500	10,000	many
	S. hemolyticus, bovine....	800	700	600	10,000+	many
	S. lacticus.....	500	10,000	many	many	many
30 C.	S. hemolyticus, human....	1,000	900	1,600	many	many
	S. hemolyticus, bovine....	400	600	1,000	6,000	many
	S. lacticus.....	500	10,000+	many	many	many
37 C.	S. hemolyticus, human....	1,200	10,000	20,000	many	many
	S. hemolyticus, bovine....	1,400	8,000	many	many	many
	S. lacticus.....	1,500	3,000	many	many	many

Two strains were used in nearly all the work discussed in this paper. One was an organism of the epidemic type isolated during the height of the Chicago epidemic from the blood of a fatal case. The other was isolated from an inflamed udder in pure culture. It showed the features of the epidemic type only after animal passage, but soon reverted to the ordinary hemolytic type. These organisms have maintained their properties and their virulence for animals unimpaired for over two years. They both ferment dextrose, lactose, saccharose and maltose, and do not ferment inulin, raffinose or mannite. Both are virulent for animals. In practically all of the following experiments

7. *Science*, 1912, 35, p. 223.

these two organisms were observed side by side, and in no instance were any differences noted in their behavior. Furthermore, though carefully sought for, no alterations or transmutations of any kind were observed in these strains of streptococci or in several other strains tested, when growing or remaining in milk or in milk products.

In order to appreciate fully the influence of temperature on growth and before presenting other tables compiled from observations under varying conditions, there are given in Table 1 the results of growing, in sterile milk, strains of hemolytic streptococci of bovine and human origin and also a strain of the streptococcus lacticus at temperatures varying from 10 to 37 C. The figures in this table and also in the subsequent tables represent the number of colonies in a standard loop grown on plates of human blood agar.

TABLE 2
GROWTH OF HEMOLYTIC STREPTOCOCCI IN COMMERCIAL MILK AT VARIOUS TEMPERATURES

Temp.	Organism	At Once	3 Hours	6 Hours	20 Hours	48 Hours
10 C.	S. hemolyticus, human....	39	55	67	72	80
	Other bacteria.....	16	24	34	23	27
	S. hemolyticus, bovine....	56	40	56	60	60
	Other bacteria.....	20	22	40	35	50
20 C.	S. hemolyticus, human....	92	97	66	260	250
	Other bacteria.....	48	116	84	1,960	many
	S. hemolyticus, bovine....	2,000	2,000	1,500	600
	Other bacteria.....	75	500	4,000	many
26 C.	S. hemolyticus, human....	8	10	?	0	0
	Other bacteria.....	2,000	3,000	10,000	many	many
	S. hemolyticus, bovine....	800	1,000	500	600	?
	Other bacteria.....	1,000	1,500	2,000	many	many
30 C.	S. hemolyticus, human....	500	600	400	few	?
	Other bacteria.....	20	30	6,000	many	many
	S. hemolyticus, bovine....	2,000	1,500	400	few	?
	Other bacteria.....	15	30	10,000	many	many
37 C.	S. hemolyticus, human....	46	106	111	3,200	120
	Other bacteria.....	9	24	16	232	10,000+

At 10 C. no appreciable increase occurs in any of the strains tested; at 20 C. at the end of forty-eight hours there is some increase in the hemolytic streptococci of human and bovine origin, but little or none at the end of twenty hours. The streptococcus lacticus, however, grows well, and even at the end of three hours has multiplied considerably. At 26 C. there is naturally greater multiplication of all strains than at 20 C., but it should be noted that the hemolytic streptococci are slow in proliferating, for, even at the end of six hours, they have not increased appreciably. At 30 C. and at incubator temperature (37 C.), rapid development of all strains takes place as would be expected. Even at 37 C., little or no multiplication occurs in milk

at the end of one or two hours, but at the end of three hours marked development is going on.

The effect of the contaminating germs in commercial milk on the growth of hemolytic streptococci, evidently of importance, was next considered. To determine this effect, hemolytic streptococci were added to ordinary commercial, pasteurized milk, the tubes being placed at various temperatures and blood agar plates inoculated at frequent intervals with comparable quantities. The results are given in Table 2. Before the addition of hemolytic streptococci, there were in the commercial milk used in this experiment 5,000 bacteria per c.c., including streptococcus lacticus, colon bacillus, bacillus lactis aerogenes and a few staphylococci, but no hemolytic streptococci.

In ordinary pasteurized milk it is uncommon to find hemolytic streptococci. In plating out samples of such milk, I have observed occasional colonies only. If the pasteurization is efficient, these germs should all be killed since they are readily destroyed, as I have repeatedly observed, at a temperature of 60 C. for twenty minutes. Their presence, therefore, probably means either inefficient pasteurization or subsequent contamination. It is perhaps possible that strains of hemolytic streptococci do exist which resist this temperature but I have not encountered such. Indeed, their thermal death point is usually considerably below 60 C.; 54 C. is given by Sternberg and others. I have not systematically examined non-pasteurized market milk for these organisms but it is known, of course, that they are found every now and then in such milk. There is considerable data in the literature on the presence of streptococcus pyogenes in milk and probably many of these strains, but not all, were hemolytic in character.

TABLE 3
EFFECT OF BUTTERMILK ON HEMOLYTIC STREPTOCOCCI (BOVINE STRAIN) AT 37 C.

	Buttermilk	Neutralized Buttermilk
0 hours	1,000	10,000
3 hours	0	1,500
6 hours	0	10,000
24 hours	0	many
72 hours	0	10,000
96 hours	0	10,000
144 hours	0	128
168 hours	0	600
192 hours	0	40
240 hours	0	2
264 hours	0	3
312 hours	0	0

It is seen from this table that at 20 C. there is little or no increase in the hemolytic streptococci, but marked increase of other bacteria (many of them streptococcus lacticus). At the higher temperatures, also, the contaminating bacteria rapidly proliferate while the hemolytic

streptococci either do not grow at all or are apparently killed. In some of the tubes, there was certainly some increase of the streptococci in the first twenty hours. Later, there was a decrease or an entire disappearance. It is not always easy to determine exactly at what point they are all killed; at times one must make many plates and many dilutions in order to isolate the streptococci. It will be seen, then, from this table that as the temperature increases, the rapid growth of the contaminating bacteria very largely checks the growth of the hemolytic cocci, which, in their absence as shown in Table 1, would develop rapidly.

TABLE 4

EFFECT OF SOURING MILK ON THE VIRULENCE OF STREPTOCOCCI

Temperature 20 C.

	S. hemolyticus	Other bacteria	Inoculation of Rabbit
At once	1,000	20	Death in 36 hours
24 hours	1,000	10,000	—
48 hours	800	Many	Death after 48 hours
72 hours	—	—	—
96 hours	400	Many	Died at once from embolism
120 hours	50	Many	No effect
148 hours	50	Many	—
9 days	None	Many	No effect

Temperature 27 C.

	S. hemolyticus	Other bacteria	Inoculation of Rabbit
At once	3,000	15	Joint lesion in 3 days.
24 hours	2,000	8,000	Death in 2 weeks
48 hours	12	Many	Joint lesion in 3 days.
72 hours	3	Many	Death in 16 days
96 hours	2 col.	Many	No effect
120 hours	—	—	No effect
148 hours	—	—	—
9 days	—	—	—

The same result is obtained also by testing the growth of hemolytic streptococci in sour milk and buttermilk which have been rendered sterile by heat. Table 3 shows the effect on streptococci of plain sterile buttermilk and of sterile buttermilk which has been brought to the neutral point or slightly beyond with NaOH. In the acid milk all the hemolytic streptococci were dead in three hours. The same milk neutralized, furnished a good medium in which they thrived for several days after which time they gradually died out. The same would probably be true of cottage cheese made with sour milk. Such rapid

destruction of cocci as indicated in this table will be obtained only if the milk has been allowed to sour at room temperature (20-22 C.) for forty-eight hours or longer after curdling.

In order to further test the effect of souring on streptococci, milk, to which these organisms had been added, was injected into rabbits at intervals as the souring process went on. In one series the milk was kept at 20 C. and another at 27 C. Bacterial counts were also made. The results are given in Table 4. It will be seen that after a relatively short interval, depending on the temperature, the streptococci in such milk lose their virulence for rabbits. During this time the hemolytic streptococci have decreased while the contaminating germs have increased markedly. The rabbits were all injected with 3 c.c. of milk. The figures in the table represent the number of organisms in one standard loop of the same milk. The injections must be made very carefully and slowly, otherwise death will occur from embolism.

The effect of butter on the viability of hemolytic streptococci was determined and the results are given in Table 5. The butter used was ordinary commercial butter purchased from a large dairy firm. To this the streptococci were added in sufficient quantity so that a standard loop contained about 5,000 organisms. The plain butter which is acid in reaction was tested, as was also some of the same butter after careful neutralization with NaOH. It will be seen from the table that in the plain butter there is a rapid decrease in the number of streptococci and a more moderate decrease in other organisms. Ordinarily, all have disappeared entirely after seventy-two to ninety-six hours. Several experiments of this kind show that there is some variation in the time of disappearance, but as a rule, it is approximately as represented in the table. In the neutralized butter the hemolytic streptococci live much longer and there may be a slight increase, but later they decrease. The other bacteria also tend to increase and in some of the experiments this increase was marked. It would seem then that a large part of the decrease of organisms in butter on standing is due to the acidity.

Both home-made and commercial ice-cream were tested with respect to the viability of hemolytic streptococci. The former was made with milk and eggs and a small amount of sugar and the streptococci were added before freezing. No hemolytic streptococci of any kind were present before, as shown by careful plating. The commercial ice-cream was purchased from a small dealer. It contained

many bacteria, but no hemolytic streptococci. Some of the frozen cream was softened and after adding the streptococci, it was frozen again. Plates were made at intervals of one to three days for eighteen days. During this time the samples of ice-cream were kept firmly frozen. The results are given in Table 6. Practically no change appeared in the number of streptococci or in the number of other organisms during the entire period. It was furthermore shown by inoculation that the hemolytic cocci retained their virulence during this period.

TABLE 5
EFFECT OF BUTTER ON HEMOLYTIC STREPTOCOCCI AT ROOM TEMPERATURE

Organism	Plain Butter				Neutralized Butter	
	S. hemolyticus, human	Other bacteria	S. hemolyticus, bovine	Other bacteria	S. hemolyticus, human	Other bacteria
At Once	5,000	76	5,000	500	5,000	50
24 Hours	3,000	10	6,000	600	6,000	100
48 Hours	80	12	—	—	—	—
72 Hours	0	12	25	Few	5,000	200
96 Hours	0	20	1	Few	8,000	300
148 Hours	—	—	0	Few	6,000	1,000
192 Hours	—	—	—	—	150	Many
240 Hours	—	—	—	—	60	Many
288 Hours	—	—	—	—	100	Many
408 Hours	—	—	—	—	0	Many

TABLE 6
VIABILITY OF HEMOLYTIC STREPTOCOCCI IN ICE CREAM

	Home-Made Ice Cream		Commercial Ice Cream	
	S. hemolyticus, human	Other Bacteria	S. hemolyticus, human	Other Bacteria
Before freezing	6,000	30	—	—
Just after freezing	5,000	50	6,000	100
24 hours	5,000	25	5,000	50
48 Hours	6,000	10	4,000	200
72 Hours	5,000	200	4,000	250
120 Hours	8,000	100	6,000	400
168 Hours	8,000	100	7,000	60
216 Hours	6,000	10	5,000	—
12 Days	6,000	4	4,000	50
15 Days	5,000	5	4,500	200
18 Days	4,000	8	3,500	—

When cream is separated from milk by centrifugal force, the hemolytic streptococci behave as other bacteria, that is, some are carried upward by the oil droplets and others are thrown down, the

result being that the cream and sediment contain many more cocci than the milk. The skim-milk is not deprived entirely of the streptococci, but contains decidedly less than the cream or the sediment. The following figures obtained by experiment fairly represent the distribution of hemolytic streptococci per 0.1 c.c. in the various portions. Whole milk before centrifugation, 2,000; after centrifugation, cream, 10,000; sediment, 50,000; skim-milk, 600. These figures in a general way, agree with the figures obtained by others as regards the distribution of bacteria of various kinds in cream and skim-milk.

The following deductions, I think, may be made from the data obtained. Hemolytic streptococci do not multiply practically at all at 20 C.; as the temperature is raised to a point where they thrive, other organisms, always present in commercial milk, grow so much more rapidly and produce acid to such an extent that the streptococci very soon not only are inhibited in their growth, but killed. Furthermore, exposure of hemolytic streptococci in milk to the optimum temperature for one to two hours will result in little or no multiplication. Hence there can be no appreciable increase of streptococci, either while milk is being cooled or in the heating or cooling of milk during the process of pasteurization. It is evident, too, that under the conditions which ordinarily prevail in the handling of milk, no matter how unsanitary, these streptococci cannot develop to any great extent; that is, streptococci coming from the hands or the throat of a milker or a milk-handler, would multiply little or not at all in the milk. This fact should be considered in its bearing on the causation of milk epidemics of sore throat. The character of these epidemics, their explosive onset, etc., suggest that the infectious organisms are probably present in large numbers and that the persons infected have probably taken large doses of the cocci. This would not so readily occur if the origin is from the hands or the throat of a milk-handler. On the other hand, it is readily explained on the assumption that the infectious agent comes directly from the cow. In the milk of cows suffering from mastitis there may be enormous numbers of hemolytic streptococci. Actual count in one instance gave 200 million hemolytic streptococci per c.c. of ropy milk as it came from the udder. This means that one pint of such milk contains 100 billion streptococci, which is about equivalent to the number of streptococci grown on 100 ordinary blood-agar slants. Of course, it is true that the cocci are not always present in such numbers

in infected milk, and furthermore, milk from a diseased cow, if it is used, is mixed with milk from the entire dairy, which means considerable dilution.

It is of course not impossible for milk to become dangerously infected by a milker or a milk-handler. Streptococci from such a source may get into the milk and if sufficiently virulent, tho few in number, may undoubtedly cause the disease. In other diseases like diphtheria, typhoid fever and scarlet fever, which at times are transmitted through milk, it is apparently true that comparatively few germs entering the milk are sufficient to transmit the disease. In seeking the source of contamination in any milk epidemic, therefore, the possibility of infection from a human carrier as well as infection from a diseased cow should always be considered. Furthermore, every investigation into the cause of an epidemic should not only look especially into these two possible sources, but should also consider other means of transmission, even tho remote or hypothetical, such as food, water, animals, insects, etc. The exact rôle of personal contact in all epidemics of sore throat should also be carefully analyzed.

SUMMARY

In the process of milk-souring the growth of hemolytic streptococci is inhibited and the organisms are gradually destroyed. They are killed in three hours or less by the acidity of sour milk (forty-eight hours after curdling) and of buttermilk. In ordinary butter they die in the course of a few days, due to the acidity, altho in neutral butter they live for a long time.

In ice-cream, hemolytic streptococci remain alive for at least eighteen days without any appreciable diminution in number or virulence. Ice-cream would seem to be, therefore, a most suitable medium for the transmission and preservation of dangerous streptococci.

Separator-cream contains more streptococci than the whole milk, skim-milk considerably less, while the sediment contains a large number.

Hemolytic streptococci do not multiply to any extent at 20 C. or below in milk; at 26 C. there is little or no multiplication during the first six hours in sterile or commercial milk, but at the end of twenty hours there is considerable increase in the sterile milk. In the commercial milk their growth is inhibited by the growth of other bacteria; this holds also for higher temperatures.

It seems that under no conditions met with in the ordinary handling of milk can there occur any appreciable multiplication of hemolytic streptococci.

Inasmuch as under the most favorable temperature, multiplication of hemolytic streptococci in milk does not occur inside of one to two hours, it is impossible for any appreciable increase to occur during the short interval between the raising and lowering of the temperature of milk in the process of pasteurization.

THE RENAL CHANGES IN RABBITS INOCULATED WITH STREPTOCOCCI *

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A number of writers have commented on the difficulties in producing chronic nephritis in animals.

Ophüls¹ says "a very serious obstacle to experiments of this kind is the spontaneous occurrence of chronic nephritis in the animals used. Even in rodents, chronic nephritis does occasionally occur spontaneously." And Pearce² says: "It must be admitted that the experimental study of nephritis supports the more common conception of the etiology of chronic nephritis in man, that is, that it is a gradually developing lesion due to long continued insidious action of some ill defined toxic substance. With the possible exception of Dickson, the results obtained have been neither constant nor of such nature as to justify the term of chronic nephritis. Occasional positive findings, in view of the frequency of spontaneous lesions, must be regarded with suspicion." Dickson³ also remarks that "a review of the literature on experimentally produced chronic nephritis forces one to the conclusion that the results obtained have not been very satisfactory."

In the article on experimental cardiorenal disease by Christian, Smith and Walker,⁴ mention is made by Smith of his examination of the kidneys of normal rabbits as controls. It is unfortunate that a more detailed account is not given by Smith of the conditions in these kidneys since his examination is one of the few that concern so considerable a number of normal rabbit kidneys. Smith's statement is: "The lesions when found consisted in almost every instance of a single area of round cell infiltration, connective tissue increase or a small group of dilated tubules." He also states that "some lesion is found in 11 of the 50 rabbits." Ophüls and Dickson⁵ have also made a careful study of the kidneys of 50 normal rabbits. These animals were partly from the market, partly animals raised in the laboratory. Many had been used in the physiological laboratory and killed immediately after experiments. Several were old rabbits. Concerning the kidneys of these rabbits he says: "Twenty-eight rabbits, among them some old ones, had entirely normal kidneys, 9 showed parenchymatous lesions, 3 a few areas of cellular infiltration. In 10 we found scattered small areas in which were marked interstitial lesions with the formation of small depressions on the surface. Four of these proved to be chronic septic foci which extended from the vicinity of the papilla to the outer surface. In the other 6 the same arrangement of the new-formed connective tissue in narrow radial bands which start deep in the pyramids was noticeable although no evidence of a septic infection could be discovered. The interstitial lesions were accom-

* Received for publication July 6, 1914.

1. *Jour. Med. Research*, 1908, 13, p. 497.

2. *Arch. Int. Med.*, 1910, 5, p. 133.

3. *Ibid.*, 1909, 3, p. 375.

4. *Arch. Int. Med.*, 1911, 8, p. 468.

5. *Proc. Soc., Exper. Biol. and Med.*, 1910-11, 8, p. 75.

panied by marked epithelial lesions in 2 cases only, producing to a certain extent the picture of a chronic parenchymatous nephritis, although the lesions were never very extensive. Lesions of the blood-vessels or primary lesions of the glomeruli were not found in any case."

Ghoreyeb⁶ does not state how many rabbits were used altogether in the experiments dealing with mechanical obstruction of the blood-supply of the kidneys. There is, however, mention of 47 and of these 8 had "spontaneous nephropathy." The changes described correspond most with those we have considered as chronic.

More recently Longcope⁷ reports the examination of the kidneys of 24 supposedly normal rabbits with the following results: "The kidneys of 16 were absolutely normal, in 4 they showed old fibrous scars in the cortex, . . . and in 4 there were one or two patches of small round cells between the tubules of the cortex—in no instance were glomerular lesions encountered."

A milk epidemic in Chicago of acute angina led to experiments⁸ that afforded good opportunity to examine the changes in the kidneys of rabbits inoculated with streptococci isolated from the patients to compare such changes with those in rabbits inoculated with other streptococci and also to study the alterations in the kidneys of rabbits injected with streptococcus vaccine and antistreptococcus serum.

The kidneys of 58 rabbits were examined. Of these 51 were inoculated with hemolytic streptococci; 6 with streptococci isolated from patients with epidemic sore throat, and 1 with streptococcus viridans. Eleven rabbits received antistreptococcic serum either before or after inoculation with living streptococci; 15 received vaccine before inoculation and 2 received both serum and vaccine. The evidences of immunity possessed by these animals have been studied by J. J. Moore,⁹ and it may be stated now that there was no particular difference in the lesions in the untreated and the treated animals except that in the latter the changes were fewer and milder.

The tissues were fixed in Zenker's fluid, imbedded in paraffin and stained with hematoxylin and eosin, phosphotungstic acid hematoxylin, polychrome methylene blue and eosin, also by the Giemsa and Wright methods for bacteria. That some statement as to the amount of renal tissue examined might be made, an account was kept of the linear measurement of the external margins of the sections examined from each block. This is readily arrived at by measuring carefully the capsular margin of one section and multiplying it by the number of sections from that block. On an average 52.3 cm. was examined from each rabbit.

Not all the protocols of the experiments need be published; the following will serve to indicate their character:

6. *Jour. Exp. Med.*, 1913, 18, p. 29.

7. *Ibid.*, 1913, 18, p. 678.

8. Leila Jackson, Experimental rheumatic myocarditis, *Jour. Infect. Dis.*, 1912, 11, p. 243, and Experimental streptococcal arthritis in rabbits, *Ibid.*, 1913, 12, p. 364.

9. *Jour. Infect. Dis.*, 1914, 15, p. 215.

Rabbit 35-D.—April 2, 1912. Injected intravenously with a 24-hour growth from one blood agar slant of streptococcus (217) isolated from pus obtained from suppurating glands of the neck; death eleven days later.

Autopsy: There are a few small hemorrhages in the endocardium; the heart valves are normal; many of the joints¹⁰ are swollen.

Streptococci in pure culture were obtained from the heart's blood and peritoneal fluid.

In a field of 4.5 mm. in diameter there are forty-six lesions in a medullary pyramid. Some are more or less coalescent, for example in one group there are seven, in another six, in a third four, but most of them are fairly discrete. At one point a number are grouped about a region of necrosis which measures approximately 0.8 mm. across, and in this there are a number of isolated masses of streptococci with rounded contours because the vessels they have occluded are cut across obliquely. In this necrotic region there is very little reaction. Nearly all of these focal lesions consist chiefly of necrotic leukocytes and bacteria. They are on an average about 0.2 mm. in diameter; some of the uriniferous tubules in and about them contain fibrin. There are very few such focal lesions in the cortex. Many of the convoluted tubules in the cortex contain casts, some of which are leukocytic.

In the cortex are acute changes in a few glomeruli with destruction more or less complete of the tuft, also in the intertubular regions the vessels are replaced in a few places by nuclear fragments—masses of material staining with hematoxylin so as to form a blue mesh for the tubules. These alterations are generally in the middle zone of the cortex and in the labyrinth.

Then, too, in the cortex, there are other alterations apparently of longer standing and consisting of perivascular collections of lymphocytes and larger cells¹¹ about the arcuate as well as the interlobar veins. All stages of emigration of the leukocytes may be seen. Where the cortex is drained by pyramids with changes like those to be described presently, many of the convoluted tubules are dilated and contain hyaline casts.

In the pyramids there are numerous focal lesions, so numerous that in the sections made through a region which (in the sections) has margins measuring 1 cm. on two of the three sides and 0.5 cm. on the third, there are from thirty to forty-five in the various sections. They measure from 30 μ in diameter to a millimeter, this last applying to dimensions parallel with the tubules. They nearly all consist of masses of nuclear debris, in a few the necrosis is characterized by karyolysis, in many bacteria are present in more or less compact masses. Many of these lesions in the pyramid are coalescent. The aggregate capsular margin is 40 μ .

Rabbit 11-M.—Oct. 26, 1912. The rabbit received intravenously one-eighth of the twenty-four-hour growth on one blood agar tube of streptococcus 256.

Oct. 28. Left shoulder joint swollen.

Nov. 3. Swelling about the left shoulder joint decreasing.

Nov. 11. Left shoulder joint markedly swollen.

Dec. 6. Less swelling of left shoulder but rabbit still limps.

Jan. 1, 1913. Killed.

10. A description of the changes observed in microscopic preparations of this joint and the joints of other rabbits of this series may be found in the *Jour. Inf. Dis.*, 1913, 12, p. 377.

11. Many of these cells are in all respects identical with the large cells met with in the myocardium and joint lesions described elsewhere (Jackson).

The cortex is not narrowed and the labyrinth and medullary ray markings are largely absent. In the outer half of the cortex there are many retention cysts—formerly tubules. They are rarely single but several, as many as three or six, occur closely together and some are in contact. The dilatation rarely exceeds twice the size of the area usually presented by sections of the convoluted tubules cut as they are at all possible angles. Some are empty but many contain an eosin staining homogeneous material shrunk away from the lining of greatly flattened cells.

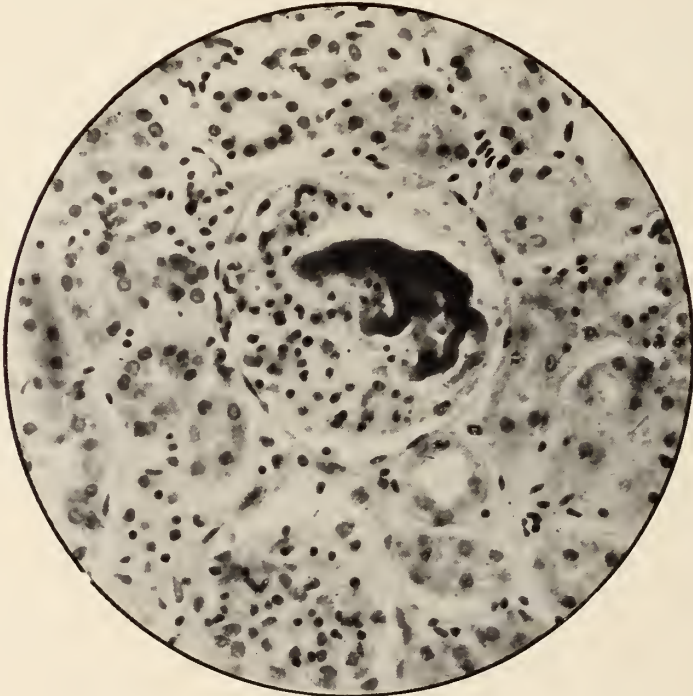


Fig. 1.—A glomerulus in which are masses of cocci filling a group of capillaries. From a rabbit dying 9 days after inoculation. $\times 325$.

The next most pronounced alteration is the presence of small groups of lymphocytes and plasma cells in both cortex and pyramids, more numerous in the latter, where in a field 4.5 mm. in diameter six to eight can be counted, in regions similar in size in the cortex the average is less than one. They are rarely larger than can be contained in the field of the immersion lens and many are smaller. Plasma cells are few, lymphocytes more numerous but most of the cells in these regions appear to belong to the kidney proper. The increase in nuclei, at least in some of these regions, is apparently dependent on the absence of tubules and consequently falling together of stroma and blood-vessels. In these regions and at many places in both pyramid and

cortex there are considerable quantities of golden brown pigment in the cells. The pigment is generally found only in the epithelial cells and in places where the changes enumerated occur. Cortical margins measure 49.4 cm.

Vaccine Rabbit 53-M.—Sept. 10, 1913. Injected subcutaneously 500 millions of killed streptococci (256); a similar amount was given on the 14th of September; on the 18th, 750 millions, on the 22d, one billion, and on the 26th, 750 millions.

Oct. 1. Injected intravenously one-tenth of twenty-four-hour growth of living streptococcus 256.

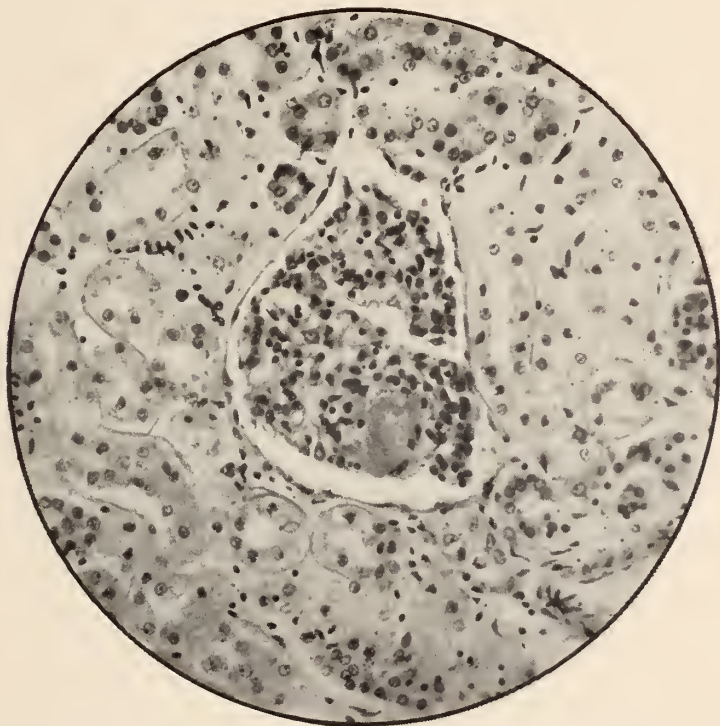


Fig. 2.—A glomerulus containing a hyaline thrombus. From a rabbit dying 7 days after inoculation. $\times 275$.

Oct. 3. No joints involved.

Oct. 4. Metacarpal joints of the right foot swollen.

Oct. 5. Right ankle slightly swollen but rabbit does not limp.

Oct. 10. Joints do not appear worse. Killed.

The lungs and heart appear normal; the spleen is cyanotic; there is coccidiosis of the liver; the kidneys are normal; the right axillary and inguinal lymph glands are enlarged; the right ankle is swollen and there are petechial hemorrhages beneath the skin and extending nearly to the knee; the right wrist is slightly swollen and the surrounding tissues edematous.

There are no changes in the sections except small collections of lymphocytes about some of the large vessels. The cortical exteriors of the sections examined aggregate 94.8 centimeters.

Vaccine Rabbit 27-M.—Jan. 6. Injected subcutaneously 500 millions of killed streptococcus 256; January 8, one billion; January 11, two billions of various strains; again on January 14 and 16; January 18, two billions streptococcus 256.

Jan. 20. The rabbit weighs 3 pounds and 5 ounces.

Feb. 10. No arthritis. Weight 4 pounds and 3 ounces.

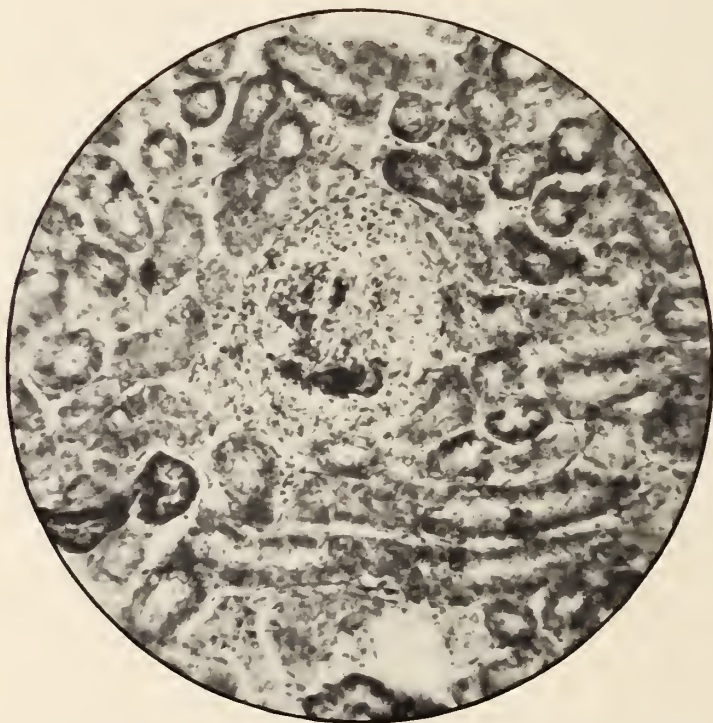


Fig. 3.—Masses of fibrin in a glomerulus. From rabbit dying 7 days after injection. $\times 200$.

March 1. Injected intravenously one-tenth of the twenty-four-hour growth on a blood agar slant of streptococcus 256.

March 3. Aborted during the night.

March 4. Appears sick but there is no arthritis.

March 5. Rabbit dead. Weight is 3 pounds and 10 ounces.

The lungs are normal; there are small hemorrhages along the tricuspid valve; the spleen is greatly enlarged, soft and cyanotic; the liver is fatty; the kidneys and adrenals are normal; the uterus contains pus and the serosa is injected. Streptococci were recovered from the heart's blood, peritoneal fluid and uterus.

There is a spot of necrosis 0.5 mm. in diameter without cellular exudate about a small group of intracapillary masses of cocci in the distal part of a medullary pyramid close to the pelvic mucosa.

In similar situations there are focal lesions 0.2 to 0.4 mm. in diameter, much more numerous, and with characteristics so different that it seems safe to assume that the agents causing them arrived at these places at different times. There are small collections of plasma cells and lymphocytes, the former predominating. The outermost tubules in these places have pyknotic nuclei; those a little nearer the centers are more or less infiltrated with leu-

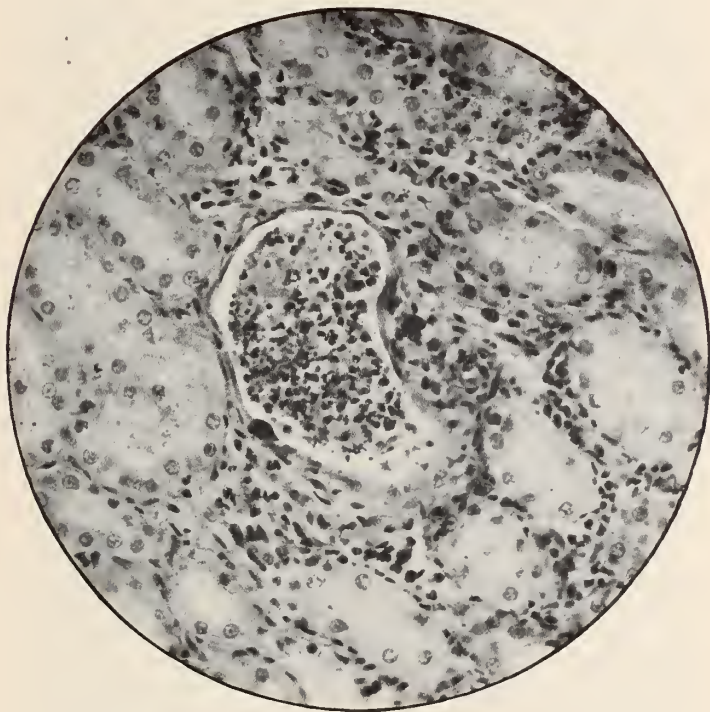


Fig. 4.—An acutely disorganized glomerulus. From rabbit dying 11 days after inoculation. $\times 250$.

kocytes, the still more central tubules are altogether replaced by the infiltrating cells. In some of the tubules on the margins there are epithelial cells with dividing nuclei. More extensive and less sharply outlined infiltrations with plasma and lymphocytes are present in the medullary pyramid close to the cortex. For example, in one section 12 lesions coalescent in varying degrees fill a place 2 by 1.6 mm.

There are many aggregations of lymphocytes about the large arcuate veins.

There are very few changes in the cortex. They consist of chronic or subacute lesions in the labyrinths in the middle of the cortex or in its outer-

most portion, the latter as minute wedge-shaped lesions. The amount of cortex examined is 49.2 cm. measured along the subcapsular margins and including all the sections examined.

Serum Rabbit 17-M.—Nov. 12, 1912. This rabbit received intravenously $\frac{1}{10}$ of the 24-hour growth from one blood agar slant of streptococcus 256.

Nov. 15. The left elbow is swollen; 2 c.c. of antistreptococcic serum was given intraperitoneally; again on Nov. 17, 19 and 20.

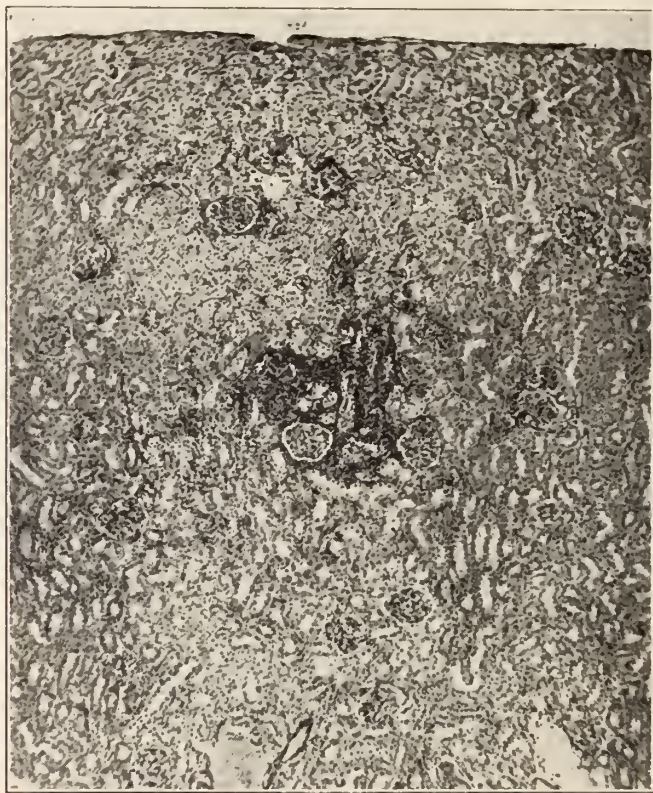


Fig. 5.—A typical subacute focal lesion in the cortex. $\times 200$.

Nov. 21. The rabbit died.

The lungs and heart appear normal; the spleen is swollen and cyanotic; the liver is enlarged and very friable; the kidneys appear normal.

There are practically no changes in 12 sections from the two kidneys; from one the sections have an aggregate cortical margin of 44.2 cm.; those from the other 21.6 cm. About some of the interlobular veins there are small collections of lymphocytes and many are of the large variety. There are also clumps in the vein channels. A few mitotic nuclei are found.

In ten, or 17 percent, of fifty-eight rabbits there were no changes in the sections examined. Acute changes were found in sixteen, or 28 percent, of the rabbits and all but two of these occurred in rabbits dying within eleven days after inoculation. The other two were treated with antistreptococcus serum and died twenty-four and thirty-two days, respectively, after inoculation and in each animal there was a

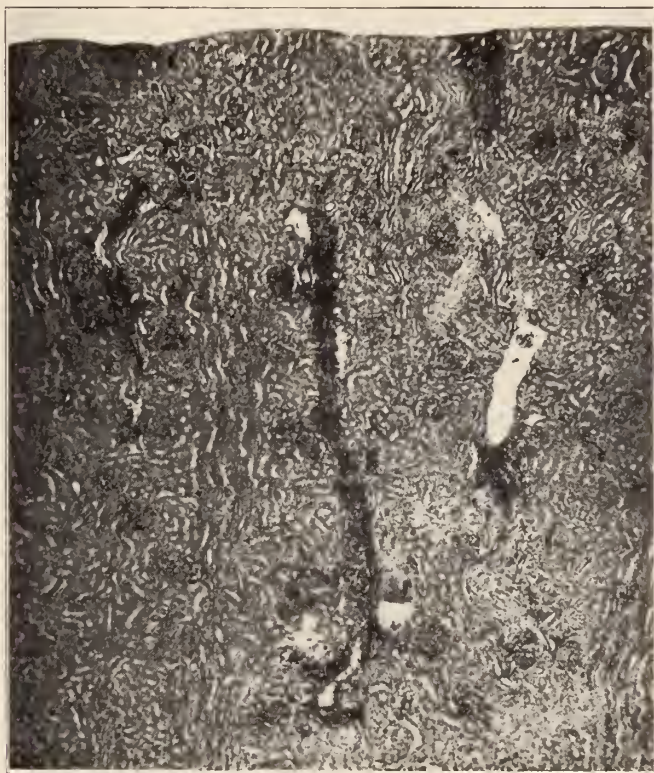


Fig. 6.—An interlobular vein surrounded by lymphocytes and plasma cells. From the kidney of rabbit dying 42 days after inoculation. $\times 35$.

mitral endocarditis. No acute changes were observed in the kidneys of eight rabbits dying of an epidemic, all of which had recovered from the streptococcus infection.

Of the acute lesions intracapillary bacterial emboli (Fig. 1) were most frequent; they were present in eleven rabbits. The number of glomeruli in which bacterial emboli are found varies greatly in dif-

ferent animals. In some, prolonged search is necessary to discover any, while in one case as many as one in every ten glomeruli contains bacterial emboli. The only explanation for the absence of changes in the glomeruli of rabbits observed for long periods is that *these acute lesions heal*. In some animals mycotic emboli occur in all parts of the kidney, in others only in the glomerular loops or in the capillaries of the cortex or medulla, in a few cases only is there necrosis or reaction of the surrounding tissue. Abscesses (Figs. 2 and 3) were found in



Fig. 7.—Two veins containing more than the normal number of large and small lymphocytes. A. A cell in mitosis. From a rabbit dying 5 days after inoculation. $\times 160$.

the kidneys of two rabbits, in one, a five-day rabbit, they were few and small and located in the medulla, in the other, an eleven-day rabbit, they were numerous and small, the largest about 0.2 mm. across, some partially coalescent, others fairly discrete, present in both cortex and medulla. Infarcts were found in the kidneys of two rabbits, and an acute glomerular nephritis in the kidneys of one dying seven days after injection. The lesions in the latter are characterized by large

masses of cocci in the glomerular loops, hemorrhages into other glomeruli or into the capsular spaces with partially or totally disrupted blood-vessels, masses of fibrin (Figs. 4 and 5) and in some glomerular lesions a more or less cellular exudate.

Subacute changes, lymphocyte and plasma cell exudates (Figs. 6, 7 and 8), were found more frequently than any other alteration, as



Fig. 8.—A more advanced subacute focal lesion producing slight pitting of the surface. From rabbit dying 16 days after inoculation. $\times 110$.

early as the third day and as late as the one hundred and thirteenth day. They were usually associated with other lesions either acute or chronic. We found them as the only change present in the kidneys of nine rabbits. These exudates are in general small, rarely larger than can be contained in the field of the immersion lens and many smaller. In

one case as many as forty are present in 1.5 sq. cm. of pyramidal substance, usually they are much less numerous. They are most often located about blood-vessels, usually veins, and most frequently around the arcuate vessels and vessels in the cortex. Less often they form rather large accumulations filling in defects in the parenchyma, some of them 2 mm. long by 0.5 mm. wide. They are composed almost



Fig. 9.—A fairly well healed linear scar extending the entire width of the cortex. From Rabbit 16M dying 10 days after injection. $\times 30$.

exclusively of lymphocytes or plasma cells or both, also large endothelioid cells are sometimes present in considerable numbers. Accumulations of plasma cells are somewhat less numerous than those of lymphocytes. In many of the kidneys in which there are exudates about blood-vessels there are in some of the veins more than the normal number of large and small lymphocytes and endothelioid cells

(Fig. 9). In these cells and also in the cells of the exudate surrounding the vessels mitotic nuclei are occasionally seen and all stages of migration of these cells through the vessel wall can be found.

Changes apparently of still longer duration were found in the kidneys of twenty-two rabbits. These were chiefly small regions where the parenchyma was absent, scars (Figs. 10, 11 and 12), dilated

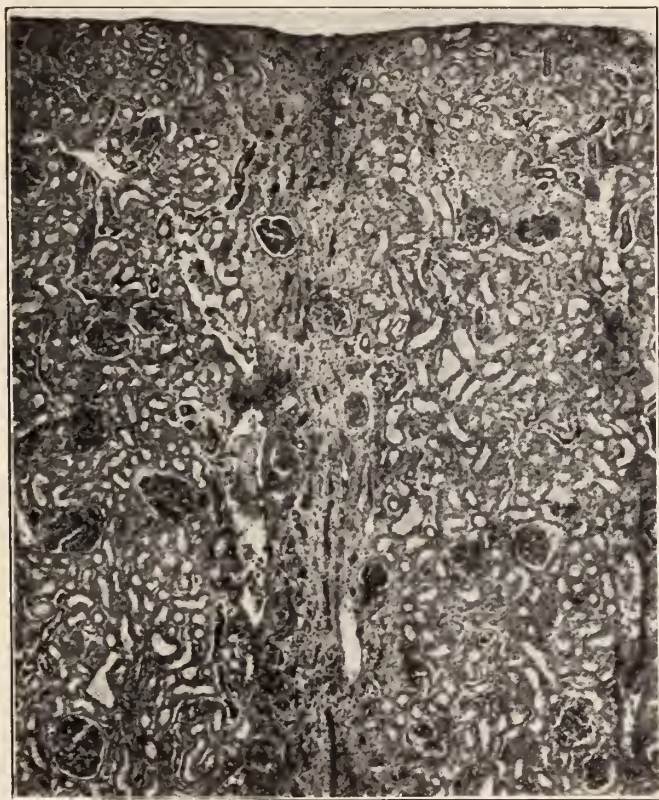


Fig. 10.—An old linear scar in the cortex of Rabbit 14M dying 32 days after inoculation. $\times 45$.

tubules with casts, retention cysts and focal regenerative changes, most of them in animals killed or dying two weeks or more after inoculation. The focal regenerative changes referred to occur in the cortex and consist of dilated tubules lined with a low epithelium sometimes empty, sometimes containing hyaline casts. The scars are usually wedge-

shaped with the base at the capsular margin producing pitting of the surface and extending into the substance for a variable distance, often the entire width of the cortex. A reasonable explanation for the absence of chronic changes in the glomeruli previously referred to is that the acute alterations are quite efficiently repaired.



Fig. 11.—A wide region of dilated tubules and retention cysts extending through the cortex into the medulla. From Rabbit 48D killed 136 days after inoculation. $\times 110$.

Changes such as these last described are the only ones we have found in the kidneys of normal rabbits; they correspond well with those described by Smith, Ophüls and Longcope. An exception concerns a glomerulo-nephritis unrelated we believe to the treatment the animal had received.

Oct. 15, 1913. Rabbit 8M was injected intravenously with half the 24-hour growth from one blood agar slant of hemolytic streptococcus 256 isolated from the tonsils of a patient with chronic rheumatism.

Oct. 16. Rabbit appears sick.

Oct. 17. Right wrist swollen and tender.

Oct. 18. Left wrist also swollen.

Oct. 19. Both ankles swollen.



Fig. 12.—Retention cysts in the cortex of Rabbit 11M killed 70 days after inoculation. $\times 35$.

Oct. 22. Left knee involved, cannot walk. Five c.c. of antistreptococcus serum injected into the knee joint, 3 c.c. into the tissues about one of the ankle joints, and 10 c.c. intraperitoneally.

Oct. 24. Died.

The cortex has an even edge with no signs of pitting. It measures 2.5 mm. thick, striations indistinct. Fully three-fourths of the glomeruli are smaller than normal by reason of thickened Bowman's capsules (Fig. 13), and probably collagenous. Of 118 glomeruli 77 had thick capsules. Such glomeruli are most numerous in the outer part of the cortex but a few are deeply located.

For the most part the thickening is three or four times greater than the normal width of the capsule, quite regular and produced by long encircling fibers or spindle-shaped cells. Most of the glomeruli close to the capsule—and there are a good many immediately beneath it—are very small, one-half or two-thirds the normal size; some are no larger than the adjacent uriniferous tubules and all have thickened capsules in varying degrees. Many of the minute glomeruli are rather closely aggregated in triangular-shaped regions, for example, in one such region 0.5 mm. in depth with a margin at the capsular edge 1 mm. long there are 16 such scarred glomeruli. In such places are many plasma cells and the uriniferous tubules remaining are greatly compressed.



Fig. 13.—Spontaneous chronic glomerulo-nephritis. A. Glomeruli with greatly thickened Bowman's capsules. $\times 60$.

The glomeruli with unchanged capsules have greatly engorged blood vessels, a few have small spaces about them in which plasma coagulated by the fixing agent has accumulated in the capsule. Some of the glomeruli with unchanged capsules are larger than normal. Small masses of fibrin are present in some of their loops. Some capsules contain nothing but red-blood corpuscles. In the medullary pyramids are quite small collections of lymphocytes or lymphocytes with a few plasma cells; they are generally in the boundary zone and so scarce that only one may be found in a section. Many of the convoluted tubules especially in the outer part of the cortex are dilated. There are no changes in the medullary pyramids.

Of the kidneys of normal animals examined by Smith, 11 of 50, or 22 per cent showed alterations; of the same number examined by Ophüls, 22 or 44 per cent, contained lesions and of Longcope's 24 rabbits there were changes in the kidneys of 8, or 33 per cent. The number of normal kidneys so far examined by us is too small to warrant a statement concerning them except that the kind of lesions found are such as we have included under chronic changes and as yet no subacute lesions have been observed.

Smith does not state in how many animals a particular kind of lesion occurs. Ophüls found areas of cellular infiltration in 3, or 6 per cent, and Longcope in 4, or 16.6 per cent, or normal rabbits.

In our experimental animals, lymphocyte and plasma cell exudates were present in thirty-two, or 56.6 per cent, from which if we deduct the larger percent (Ophüls — 4 per cent) as possibly of other origin, there still remains a considerable number resulting probably from the action of streptococci. The kidneys of twenty-two, or 38.6 per cent of the streptococcus rabbits contained chronic changes. Since the majority of the lesions observed by Ophüls, also half of those described by Longcope and all of the changes so far observed by us in normal animals, belong to this type, they can with much less certainty be attributed to the inoculation. If, however, the period of time elapsing after the injection of streptococci into the rabbits from which we examined the kidneys be arbitrarily divided into two periods, one so short that it is fair to assume that sufficient time had not elapsed for chronic changes to develop, and a second period during which such changes are possible, we may arrive at some conclusion as to whether some at least of the more chronic lesions are due to streptococci. Eight, or 25 per cent, of the thirty-three rabbits killed or dying within the first two weeks after inoculation have chronic changes in the kidneys, while the kidneys of fifteen, or 62.5 per cent, of the twenty-four rabbits living for fifteen to one hundred and eighty-six days after inoculation possess similar alterations. This rather considerable difference in the relative number of kidneys affected with chronic lesions suggests that streptococci may have been the etiologic factor in some instances. Eight animals died of the epidemic referred to. We have been unable to attribute any of the changes mentioned to it, because the changes in the kidneys of these animals were in two of them chronic, in two others subacute and in the remainder no changes were found.

A careful consideration of all of the changes has impressed us with the large percentage of lesions which we have called subacute.

Faulhaber¹² in his search for bacteria in the kidneys of persons dying of acute infectious diseases found focal accumulations of round cells in the

outermost layers of the cortex about the glomeruli and blood vessels in 33 of 55 kidneys. He attributes these to the focal presence of bacteria. In his cases of streptococcus infections when these focal lesions were absent bacteria were few.

All writers on scarlatinal nephritis have described round cell infiltration in the kidneys. Councilman,¹³ who has studied this form of nephritis most carefully, gives the location of the cell accumulations in severe cases as most frequently beneath the capsule and at the bases of the pyramids, in less marked cases as being most numerous and largest at the bases of the pyramids, occasionally forming large masses around the interlobular vessels and less frequently beneath the capsule around the stellate veins. He also observes that these cells, lymphocytes and plasma cells, are found in the vessels as well as in the tissue and that nuclear figures are sometimes found in them. Councilman believes the cells in these exudates must come from the blood vessels.

Schridde,¹⁴ discussing the origin of small cell infiltrations in the kidneys of persons dying of scarlet fever and diphtheria, observes that about the 5th day the capillaries of the medulla contain enormous numbers of lymphocytes, that by the 8th day lymphocytic infiltration is present in both cortex and medulla, derived from emigrated lymphocytes and also containing plasma cells.

Unna¹⁵ and others hold a different opinion regarding the origin of plasma cells. They believe that plasma cells arise from the proliferation of existing cells in the connective tissue.

Lymphocyte and plasma cell exudates in the kidneys of experimental animals have been described by many authors. In 1899 Bonsdorff¹⁶ mentions aggregations of round cells about destroyed tubules and about blood-vessels in the kidneys of rabbits used in which he studied elimination of streptococci. The rabbits on which these observations were made died at intervals varying from $1\frac{1}{4}$ to $6\frac{1}{2}$ days after in inoculation.

Lyon¹⁷ describing the conditions found in the kidneys of rabbits injected with diphtheria toxin makes the following statement: "In the great majority of animals which survive beyond the fourth day there is an accumulation of round cells around some of the cortical vessels. Similar accumulations do not occur in the medulla. This infiltration is always local in character and the degree is variable. Normally there is no layer of lymphoid cells around the large cortical vessels of the rabbit's kidney."

Ophüls¹⁸ notes their presence at the dividing line of cortex and medulla in the kidneys of rabbits treated with bichromate of potash.

Dickson¹⁹ injected guinea-pigs with uranium nitrate; concerning lymphocytic exudates in one of his series he says: "There were distinct areas of round cell infiltration in every case, occurring chiefly near the larger veins at the inner margin of the cortex, but in two cases there was definite infiltration in the regions of damaged glomeruli."

Smith²⁰ injected rabbits with uranium nitrate and observed that microscopically the most frequent variation from the normal was round cell infiltration, usually small, sometimes of considerable size, the location of which was variable but most often in the cortex near the periphery.

13. *Jour. Exper. Med.*, 1898, 3, p. 393.

14. *Ziegler's Beitr.*, 1913, 55, p. 345.

15. *Virchow's Archiv*, 1913, 214, p. 321.

16. *Ziegler's Beitr.*, 1899, 25, p. 188.

17. *Jour. Path. and Bact.*, 1903-4, 28, p. 430.

18. *Jour. Med. Research*, 1908, 13, p. 497.

19. *Arch. Int. Med.*, 1909, 3, p. 375.

20. *Ibid.*, 1911, 8, p. 468.

Harvey²¹ refers to round cell infiltration around damaged glomeruli and tubules in the kidneys of rabbits injected intravenously with para-hydroxy-phenylethylamin. Lymphocytic infiltration in any part of the kidney is noted by O'Hara²² in rabbits injected subcutaneously with uranium nitrate and intravenously with colon bacilli.

Longcope²³ gives the following account of round cell exudates in the kidneys of rabbits subjected to repeated proteid intoxication. "In the smallest areas the surrounding capillaries were filled with small mononuclear cells. In larger areas a single collecting tubule or group of convoluted tubules formed the center of a dense area of round cell infiltration . . . Collections of round cells were observed about the vessels in the intermediate zone and sometimes surrounding the vessels as they ran into the cortex." The preceding he regards as an early stage in the process. Of a more advanced stage he observes: "Masses of round cells in enormous numbers infiltrated the intertubular tissue extending in rays from the intermediate zone toward the capsule, occurring in great patches in the papilla." Concerning the origin he believes "it is probable that these round cells come from the circulating blood and not from the fixed connective tissue cells of the kidney, for in a number of sections the capillaries surrounding small foci of necrosis were seen to be filled with small mononuclear cells."

As previously stated the more chronic changes found in the kidneys of rabbits inoculated with streptococci are quite similar to those we have found in the kidneys of normal rabbits and also described by others.

Dickson, Ophüls, Harvey, O'Hara, Smith and Longcope believe they have to a greater or less degree, and by various means, produced a chronic nephritis in laboratory animals. The changes described by these writers are in general quite similar and correspond very well with those found in our series with the exception of the rather marked glomerular changes described by Dickson in the kidneys of guinea-pigs injected with uranium nitrate and the similar though less marked changes observed by Longcope in the kidneys of rabbits receiving repeated intoxicating doses of proteid.

In conclusion it is evident that the study of experimental nephritis in animals should be controlled by a thorough study of the kidneys of normal animals kept under similar conditions. We also believe that the best results will be obtained by the maintenance of the best sanitary surroundings for both sets of animals.

SUMMARY

Among the results are the demonstration of fewer changes in the kidneys of rabbits treated with antistreptococcus serum or vaccine than in those of untreated animals, and the presence of changes char-

21. *Jour. Path. and Bact.*, 1911-12, 16, p. 95.

22. *Arch. Int. Med.*, 1913, 12, p. 49.

23. *Jour. Exp. Med.*, 1913, 18, p. 678.

acteristic of chronic nephritis in a large number of animals inoculated with streptococci.

The most important result, however, is the experimental production of alterations essentially subacute and quite like the acute interstitial nephritis in human kidneys caused by the acute infectious diseases complicated by or due to streptococcus infection. Then too, we wish to emphasize the great need of thorough study of the kidneys of control animals in any investigation of experimental nephritis.

THE ETIOLOGY OF PYEMIC ARTHRITIS IN FOALS *

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This disease is common to calves and foals, occurring generally when the animal is but a few days old, altho typical cases may occur in foals at the age of several months. The symptoms consist of rapidly developing articular inflammation causing impaired locomotion, fever, omphalophlebitis and often pulmonary and intestinal disturbances. Suppuration of infected joints with internal abscesses is common.

Lack of definite knowledge in regard to the etiology is apparent when one studies the literature. The earliest description of the disease was by Bernard of Boulogne in 1828. The symptoms are accurately recorded and it is interesting that he believed the disease originated in the mares and was transmitted through the milk. A few years later, Prichard, an English writer, after describing the disease, states the cause to be due to an alteration in the milk, the result of working the mare and allowing the colt to suckle between periods. Other early writers considered the disease as tuberculous or as rheumatism. To-day most of those who have studied the disease believe that it results from umbilical infection at the time of birth or subsequently. This view has much evidence in its support, but altho it has been held for years and appropriate precautions taken to prevent infection through this channel, the disease is still extremely prevalent. Cases also develop in utero or apparently a few hours after birth. At present there is no unity of opinion as to the causative agent.

PERSONAL OBSERVATIONS

During the last two years many bacteriological examinations have been made of material from cases of this disease forwarded to this laboratory. In most cases practitioners would send only pus formed in the joints during the latter period of the disease and in almost all such cases the cultures showed mixed infection. That the organism to be discussed was not recovered earlier is, I think, accounted for in this

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way. On several occasions, however, I have obtained fluid from infected joints during the early days of the infection, and in this fluid the organism to be described was obtained in pure culture.

Case 1.—Colt foaled in open; disease never observed on the premises before altho three cases occurred this season; navel not ligatured at time of birth. When 8 days old developed typical case of "joint ill," the left hock being chiefly involved. Ten days after first symptoms the hock was aspirated and cultures made of the synovia; at the same time blood cultures were made from the jugular on glucose liver broth.

On the blood agar inoculated with the synovia a pure culture of a gram-negative bacillus was obtained; eight colonies developed, all of the same type. Direct smear showed a few leukocytes but no bacteria. This, however, is not strange as the inflammation is chiefly periarticular.

In eighteen hours large quantities of gas formed in the glucose broth culture from the blood. Smears showed numerous gram-negative bacilli. On plating out, a pure culture was obtained of the same organism as that from the synovia. This animal died about four weeks later and at that time I isolated streptococci as well as gram-negative bacilli, the streptococci predominating.

Case 2.—Colt, foaled in the open; navel ligatured by veterinarian and treated antiseptically. Twelve days after birth typical symptoms of joint ill were observed. Ten days later the affected hock was aspirated and cultures made from synovial fluid, blood cultures being made at the same time. From the joint on blood agar a pure culture of a gram-negative bacillus was obtained; again the number of colonies were few, about twelve, all of them having the same appearance. From the blood pure culture of an organism identical with the one obtained from the synovia was isolated.

Case 3.—Colt born in open, navel not ligatured, no evidence of umbilical infection. Ten days after birth joint ill developed, both hocks being involved, giving pure cultures of a gram-negative bacillus. This colt made a slow recovery.

Case 4.—In this case no history was given save that the sample of synovia has been aspired two hours after death. Numerous short gram-negative bacilli with many short-chain streptococci were present in the smears. From blood agar plates both organisms were isolated.

Case 5.—No history, fluid aspirated from joint after death. The smears gave gram-positive cocci and gram-negative bacilli. Culture on agar gave a growth of the staphylococcus aureus, streptococci and colonies of a gram-negative bacillus.

Case 6.—No history; blood culture sent with statement that it was from a case of septic arthritis. Blood agar plates gave pure growth of a gram-negative bacillus.

In two other typical cases, pure cultures of streptococcus were obtained from the pyemic joints. In one case this organism was obtained from the blood also.

Pus from about fifteen discharging joints and navels has been examined and all showed mixed infection with streptococci, staphylococcus aureus and colon bacilli, streptococci generally predominating.

MORPHOLOGICAL AND CULTURAL CHARACTERISTICS OF
THE BACILLUS

It is a very short bacillus with rounded ends frequently resembling a coccus, from .2 to .5 micron in width, and .3 to 1 micron in length. In old cultures much longer organisms are often observed; frequently seen in pairs, but never in chains. It is gram-negative, stains well with all anilin dyes, and very motile, especially in young broth cultures.

Agar Slants.—On agar +6 to +1.0 to phenolphthalein the growth, especially when blood has been streaked over the surface, is characteristic within twenty-four hours. The growth is dull and if it has spread to water of condensation, this is covered with a grayish pellicle; at the base of the slant numerous wrinkles develop especially in the median line. The growth is not slimy but more membranous, being removed from the slant at times with difficulty.

TABLE 1
THE RESULTS OF TESTS OF CARBOHYDRATE FERMENTATION

Strain	Glucose	Lactose	Mannite	Dulcitol	Raffinose	Saccharose	Milk
Blood, Case 1	A+ G+	A— G—	A+ G+	A+ G	A— G—	A— G—	A— G—
Joint, Case 1	A+ G+	One bubble on 2d day	A+ G+	A+ G	A— G—	A— G—	A— G—
Joint, Case 1 at death	A+ G+	A— G—	A+ G+	A+ G	A— G—	A— G—	A— G—
Joint, Case 2	A+ G+	One bubble on 2d day	A+ G+	A+ G	A— G—	A— G—	A— G—
Blood, Case 2	A+ G+	A— G—	A+ G+	A+ G	A— G—	A— G—	A— G—
Joint, Case 3	A+ G+	A— G—	A+ G+	A+ G	A— G—	A— G—	A— G—
Joint, Case 4	A+ G+	A— G—	A+ G+	A+ G	A— G—	A— G—	A— G—
<i>B. coli communis</i> "Dunham"	A+ G+	A+ G+	A+ G+	A+ G	A+ G+	A+ G+	A+ G+

A+ = acid produced. A— = no acid produced. G+ = gas produced. G— = no gas produced.

Agar Streak.—This is also characteristic after a few days' growth. Where the growth is most luxuriant at the bottom of the slant there is a tendency to spread out in concentric rings through which radiate numerous wrinkles.

Agar Plates.—At first the colonies are transparent and delicate, but in twenty-four hours a peculiar concentric ring formation occurs.

Gelatin Stab.—No liquefaction.

Milk.—Changed in reaction after five days from plus 1.4 to phenolphthalein to plus 0.9; alkaline to litmus.

Broth.—For first twenty-four hours the growth is very turbid; later some strains produce a pellicle while others do not. In both cases a slightly viscid sediment is produced.

Peptone Solution.—No indol.

Nitrate Broth.—Not reduced.

Carbohydrate Fermentation.—The media were prepared according to the standard methods of the American Public Health Association. The broth was incubated for four days. The results are given in Table 1.

From these characteristics it is seen that the organism belongs to the colon-typhoid group and is closely related to *B. paratyphosus beta*.

To further ascertain this relationship, complement fixation tests were made, using antigens prepared from two strains of the paratyphoid bacillus B., the organism under discussion and colon bacilli. The antigens were made as described on page 5 and their strengths equalized as nearly as possible. Fixation of complement was never obtained with these antigens, altho controls both positive and negative were quite satisfactory. Altho shown by these tests not to be a true paratyphoid bacillus B., the organism can, I think, be considered as of a closely related species.

AGGLUTINATION

A potent agglutinative serum was obtained by injecting a rabbit intravenously on three successive days with 50 million dead organisms (Strain 4). On the sixth day after the last injection the rabbit was bled, serum separated and inactivated at 56 C. for thirty minutes. It now caused agglutination of Strain 4 in a dilution of 1 to 3,000. Table 2 shows the results of these tests. The different strains were grown for twenty-four hours at 37 C. and broth + 10 to phenolphthalein diluted to a uniform turbidity was used as in the typhoid agglutination test.

TABLE 2
AGGLUTINATION

Strain	Dilution of Immune Serum			
	$\frac{1}{1500}$	$\frac{1}{2000}$	$\frac{1}{3000}$	$\frac{1}{4000}$
1	++	++	±	—
2	++	++	±	—
3	++	++	++	±
4	++	++	++	—
5	++	±	±	—
6	++	++	±	—
7	++	++	±	—

++ = strong agglutination. ± = doubtful agglutination. — = no agglutination.
Normal rabbit serum in no case agglutinated in dilutions above 1 to 10.

The tests, if not demonstrating the absolute similarity of these organisms, indicate their close relationship, the results being far more pronounced than those in group reactions.

COMPLEMENT FIXATION

Experiments were made to establish if possible relationship between the organism and the disease. The time of the year (November) was unfortunate, as no acute cases could be obtained and only few chronic ones. There were, however, some cured cases and the blood was obtained from them, also from their dams and from mares that had given birth to foals which developed the disease.

TABLE 3
TITRATION OF COMPLEMENT AND HEMOLYSIN

5 Percent Suspension of Sheep Cells	Hemolysin 1/1000	Complement 1/10	Normal Salt Solution	Hemolysis
.5 c.c.	.25	.25	1.5	Complete
.5 c.c.	.2	.25	1.55	Complete
.5 c.c.	.15	.25	1.6	Complete
.5 c.c.	.1	.25	1.65	Partial
.5 c.c.	.05	.25	1.7	None
.5 c.c.	.25	.2	1.55	Complete
.5 c.c.	.2	.2	1.6	Partial
.5 c.c.	.15	.2	1.65	Partial
.5 c.c.	.1	.20	1.7	None
.5 c.c.	0	0	2	None
.5 c.c.	.5	0	2	None
.5 c.c.	0	.5	1.5	None

Hemolysin .15 = 1 unit of hemolysin with .25 complement; in test .3 hemolysin would be used with .5 complement. The quantities in Tables 3, 4 and 5 refer to c.c.

Agar slants were grown for twenty-four hours, the organisms washed off in normal salt solution, the suspension heated at 60 C. for fifteen minutes, carbolic acid added to make 0.5 percent strength. Antigen was tested for specificity by using normal and immune rabbit serum and normal horse-serum. Complement was obtained in the usual way from fresh guinea-pig blood, diluted to one-tenth, then titrated. The serum to be tested was removed from the clot and inactivated at 56 C. for one-half hour within forty-eight hours after clotting. The night before the test was made sheep blood-cells (2 drops solid cells) were added to each tube and kept in ice-chest. This has been found the simplest way to avoid inaccuracy due to presence of natural hemolysin. Tables 3, 4 and 5 illustrate the methods used.

In some cases fixation could be obtained when much greater dilutions of antigen and serum were used. It must, however, be borne in

mind that the animals from which the blood was obtained were either cured cases or apparently normal animals. On this account the concentration of antigen and serum in the tests was increased, altho not sufficiently to make the test of doubtful value.

TABLE 4
TITRATION OF ANTIGEN

Antigen	Serum	Complement	Normal Salt Solution	Hemolytic Amboceptor	Sheep Cells 4 Percent Suspension	Hemolysis
1.0 c.c.	Normal	.5	—	.5	.5	Complete
.8 c.c.	.15 c.c. Normal	.5	.05	.5	.5	Complete
.5 c.c.	.15 c.c. Normal	.5	.35	.5	.5	Complete
0	.15 c.c. Normal	.5	.7	.5	.5	Complete
1.0 c.c.	.3 c.c.	.25	.25	.5	.5	Complete
1.0 c.c.	—	.5	.5	.5	.5	None
.5 c.c.	Immune .1	.5	.4	.5	.5	None
.4 c.c.	Immune .1	.5	.5	.5	.5	None
.3 c.c.	Immune .1	.5	.6	.5	.5	None
.2 c.c.	Immune .1	.5	.7	.5	.5	Trace
.1 c.c.	Immune .1	.5	.8	.5	.5	Almost Complete
—	Immune .2	.5	.8	.5	.5	Complete

Antigen, serum, complement and salt solution at 37 C. (water bath) for 30 minutes before adding amboceptor and sheep corpuscles. 0.5 per cent. antigen was maximum quantity used; 2½ times that quantity which gave complete fixation with known positive serum. This quantity was not anticomplementary nor was it hemolytic.

TABLE 5
ILLUSTRATION OF COMPLEMENT FIXATION TEST

Antigen	Serum	Complement 2 Units	Salt Solution	Hemolysin 2 units	Cells	Hemolysis
.5	Known positive .15	.5	.35	.5	.5	None
.5	Known positive .1	.5	.4	.5	.5	None
.5	Known positive .05	.5	.45	.5	.5	None
.0	Known positive .3	.5	.7	.5	.5	Complete
.5	Known negative .15	.5	.35	.5	.5	Complete
.5	Known negative .10	.5	.4	.5	.5	Complete
.5	Known negative .05	.5	.45	.5	.5	Complete
.0	Known negative .3	.5	.7	.5	.5	Complete
.5	Serum to be tested .15	.5	.35	.5	.5	None
.5	Serum to be tested .10	.5	.4	.5	.5	None
.5	Serum to be tested .05	.5	.45	.5	.5	Partial
.0	Serum to be tested .3	.5	.7	.5	.5	Complete

The results obtained from a considerable number of tests (Table 6) indicate that a specific relationship exists between the organism isolated and used as antigen and the disease pyemic arthritis or joint ill as it occurs in this region. Certain cases (2, 3, 15, 16, 17, 18) are especially interesting, positive reactions being obtained from the blood of both dam and foal. Numbers 8, 9, 25 and 29 also are worthy of

special notice, these mares having aborted during the year, and it will be noticed, gave a certain amount of fixation indicating a possible relationship between the organism isolated and that of contagious equine abortion.

TABLE 6
RESULTS OF TESTS FOR COMPLEMENT FIXATION

1. Normal horse	No fixation
2. Dam in Case 3	Complete fixation
3. Foal in Case 3	Complete fixation
4. Dam in Case 4	Complete fixation
5. Gelding, 12 years, on infected farm	Complete fixation
6. Mare, 5 years, had arthritis as foal	No fixation
7. Dam of diseased foal	Partial fixation
8. Mare, aborted in spring	Complete fixation
9. Mare, aborted in spring	Complete fixation
10. Colt recovered from joint ill	Complete fixation
11. Mare, history unknown	No fixation
12-13. Geldings, history unknown	No fixation
14. Immunized rabbit	Complete fixation
15. Mare, foal with joint ill in spring	Complete fixation
16. Colt of Mare 15	Complete fixation
17. Mare, foal joint ill in spring	Complete fixation
18. Foal of Mare 17	Complete fixation
19. Colt, 2 years, pyemic	Complete fixation
20-24. Mares giving birth to foals with joint ill this spring ..	Complete fixation
25. Mare, aborted this spring	Almost complete fixation
26. Mare, never in foal, joint ill on farm	Almost complete fixation
27. Mare, had foal with joint ill all this spring	Almost complete fixation
28. Colt, joint ill one year ago	50 percent hemolysis
29. Mare, aborted two months ago	50 percent hemolysis
30-46. Mares giving birth to foals with joint ill this spring ..	No fixation
46-52. Mares that recently aborted	No fixation
38-39. Foals recovered from joint ill	No fixation
40-50. Supposedly healthy mares	No fixation

Cases 20-50 occurred in a district where septic arthritis has been prevalent for years.

DISCUSSION

To what extent the organism described is responsible for septic arthritis cannot as yet be stated. Isolating the organism from blood and joint in pure culture from acute cases, altho suggestive, by no means demonstrates that it possesses pathogenic properties for the animal from which it was recovered. But the fact that positive fixation tests have been obtained frequently with blood of diseased foals and their dams and not from the healthy controls is strong evidence in favor of a definite relationship between the organism and certain cases of arthritis. Further, I think that these results strongly support the idea that the foal frequently is infected before birth. Intra-uterine infection alone can account for the cases that develop within a few hours after birth. I would not wish to belittle the present prophylactic methods, but rather supplement them, and thereby prevent infection from a hitherto neglected source. In many instances arthritis no doubt may arise from navel infection with the usual pus-forming bacteria

subsequent to birth ; but as before stated, such infection cannot account for all cases. The possibility of infection being carried from diseased to healthy mares by coitus must not be overlooked.

I would like to draw attention to the interesting fact that the organism isolated and described in this paper bears a very close relationship to one recently isolated by Good in contagious equine abortion. In cultures the only difference observed is that the bacillus of equine abortion causes fermentation in raffinose while my organism does not attack this carbohydrate. However, difference in strains would readily account for this. The most convincing evidence that these organisms are but strains of same species is that they cannot be differentiated by means of the complement fixation test. Serums giving complete fixation with antigen prepared from my organism also give fixation in corresponding dilutions with antigen prepared from the bacillus of equine abortion. With characteristics that correspond so closely, these organisms appear to be but strains of the same species and it is quite probable that the same organism may be responsible for both septic arthritis and abortion. These diseases commonly co-exist in the same locality ; furthermore, it is of frequent occurrence for a mare to abort one season and during the next to deliver a colt that develops septic arthritis. The more one studies the clinical history of these diseases the more convincing becomes the probability of their common etiology in many instances.

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THE METABOLISM OF SAPROPHYTIC HUMAN TUBERCLE BACILLI IN PLAIN, DEXTROSE, MANNITE, AND GLYCERIN BROTHS STUDIES IN ACID-FAST BACTERIA. I*

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One of the noteworthy contributions to the chemistry of the tubercle bacillus was the discovery by Theobald Smith¹ that human tubercle bacilli produce a terminal acid reaction in nutrient glycerin broth, contrasting in this respect with bovine tubercle bacilli, cultures of which tend to an alkaline reaction in the same media. Attention was directed by him to slight variations in this reaction of the human bacilli; if the medium is not favorable for the luxuriant growth of these organisms, this terminal acidity may be lessened or even be absent. Furthermore, as he pointed out, this acid reaction is developed only when the glycerin content of the broth is 1 percent or more; a concentration of at least 2 percent of glycerin is necessary for the maximum acidity. Again, this acidity is a differential acidity, for alkaline products appear to be formed in glycerin broth coincidentally with the

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1. Trans. Assn. Am. Phys., 1903, 18, p. 108; Jour Med. Research, 1905, 13, p. 253; Am. Jour. Med. Sci., 1904, 128, p. 216.

acid products derived from the glycerin. A differential excess of acid, rather than a purely fermentative decomposition of glycerin, is responsible for this noteworthy difference between the human and bovine types of tubercle bacillus. The latter organism does not appear to form acid from glycerin. The development of alkalinity, probably derived from protein constituents of the broth, is brought out sharply when bovine and human bacilli, respectively, are grown in broth cultures containing no glycerin. In this medium, altho the growth is less luxuriant than in glycerin broth, the reaction becomes progressively alkaline in both instances.

This work is important from another point of view. Prior to the publication of these studies, investigators, almost without exception, assumed that tubercle bacilli could not be cultivated in artificial media unless glycerin was present. Some observers have gone so far as to state dogmatically that glycerin was a *sine qua non* for the artificial cultivation of these organisms. The observations mentioned above show definitely that the majority of strains of tubercle bacilli will develop, altho much less luxuriantly, even in plain nutrient broth. These organisms, therefore, are not obligately glycerophilic.

Dextrose, or muscle sugar, appears to play no part whatsoever in the development of tubercle bacilli in glycerin broth, for the amount of dextrose remains the same in broth after prolonged cultivation of these organisms in it as in uninoculated broth, as shown by the fermentation test with the colon bacillus. These observations were soon confirmed, and they were apparently accepted without question by Koch as the only practical cultural differentiation between human and bovine bacilli.

From time to time strains of tubercle bacilli have been isolated from man which appear to be intermediate between the human and bovine types when judged by this criterion, but for the most part subsequent investigation has shown that these few intermediate strains revert, after prolonged cultivation, to one type or the other, or they have been definitely referred to one of the other types by careful animal experiments. A few strains, however, appear to be exceptional in this respect.

The experiments here recorded were undertaken with the specific purpose of studying the gross metabolism of tubercle bacilli in artificial media, particularly with reference to the influence of glycerin on the metabolism of these organisms.

The organisms studied in this connection were two strains of rapidly growing human tubercle bacilli; one of these, "W", was kindly sent us by Professor Wherry of the University of Cincinnati; the other, "597", from the Museum of Natural History, New York, by Professor Winslow. Culture "W" is a descendant of a culture from Koch's laboratory, brought to this country by Professor Vaughan of the University of Michigan. It is avirulent for guinea-pigs. Culture "597" will produce tuberculosis in guinea-pigs if relatively large doses are injected intraperitoneally. It should be emphasized that these cultures, particularly "W", in virtue of their avirulence for guinea-pigs, might be questioned with respect to their identity as human tubercle bacilli.

Further observations along similar lines with human bacilli of known virulence, however, which will be presented later, have given reactions qualitatively identical with those mentioned above, and the study of a considerable series of acid-fast bacilli, not tubercle bacilli, have shown unmistakable cultural differences which appear to separate them from these avirulent tubercle bacilli.

Rapidly growing tubercle bacilli offer advantages for chemical study which are obvious; their relatively active development leads to an early accumulation of decomposition products in measurable amounts, and these products are less influenced by parallel recessive changes due to autolysis of the bacilli themselves than appears to be the case with the highly virulent, slowly growing strains.

The media selected for this study comprise nutrient sugar-free broth made from meat juice as a basis. Portions of this broth were re-enforced by 1 percent dextrose, 1 percent mannite, and 3 percent of glycerin, respectively, as additional sources of carbon. As in previous experiments,² several flasks of the same size and shape [250 c.c. Erlenmeyer flasks], each containing 100 c.c. of the medium, were prepared at the same time and inoculated and incubated under similar conditions of temperature and moisture. The methods of analysis used have also been described in detail elsewhere³ and need not be referred to here. The determinations comprise: changes in reaction, using alizarin, neutral red, and phenolphthalein as indicators; and the production of ammonia and a parallel study of the morphology, using the Ziehl-Neelsen stain. The stains for a given series were all made at the same time and subjected to the same degree of heat, decolorization, etc. to obviate errors in this direction as far as possible. The results based on the initial volume of 100 c.c. of media follow, the reaction being expressed in terms of cubic centimeters of normal acid or alkali per hundred cubic centimeters of broth, the ammonia as milligrams of nitrogen per 10 c.c. of broth. Several parallel experiments, some in duplicate, were made at different times in different lots of media. These are included to indicate the approximate limits of variation which may be expected.

The only noteworthy features in the morphology and staining reactions were a change about the end of the fourth week from the typical,

2. Kendall, Day and Walker, *Jour. Am. Chem. Soc.*, 1912, 35, p. 14.

3. Kendall and Farmer, *Jour. Biol. Chem.*, 1912, 12, p. 13.

rather long, slender rod to a short, thick rod, and a moderate proportion of non-acid-fast rods, typical, however, morphologically during the first two weeks of growth. These short, thick rods resemble strikingly the bovine bacillus. The experiments, however, do not suggest that a further cultivation of these short rods would result in a permanent change in morphology from organisms resembling the human type of the bovine bacillus to that of the bovine. The change from partial acid-fastness to complete acid-fastness seems to be some-

TABLE 1
METABOLISM OF TUBERCLE BACILLI IN BROTH

Bacillus tuber- culosis	Days	Plain Broth					Dextrose Broth				
		Alizarin	Neutral Red	Phenol- phthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenol- phthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
597	4	-1.10	0.00	-0.20	5.6	2.50	0.00	0.00	-0.20	4.9	2.50
	6	-2.50	-0.50	-0.50	10.5	4.69	-0.20	0.00	-0.10	5.6	2.85
	13	-3.40	-1.20	-0.90	26.6	11.87	-1.80	-0.50	-0.10	23.1	11.80
	20	-3.00	-1.40	-1.10	21.0	9.37	-3.20	-1.50	-0.80	30.8	15.70
	27	-3.30	-1.50	-2.00	15.4	6.88	-2.60	-1.70	-1.00	21.7	11.60
	34	-2.10	-1.50	-0.90	16.1	7.23	-1.70	-1.20	-0.90	15.4	7.85
W	4	-1.10	0.00	-0.30	2.8	1.25	-0.20	0.00	-0.20	4.9	2.50
	6	-2.30	-0.40	-0.50	8.4	3.75	-0.20	0.00	-0.20	4.9	2.50
	13	-2.80	-1.10	-0.80	25.2	11.25	-2.70	-1.20	-0.50	23.1	11.80
	20	-3.20	-1.50	-0.90	26.6	11.88	-2.80	-1.50	-0.80	23.1	11.80
	27	-2.90	-1.40	-0.60	23.8	10.63	-2.10	-1.30	-0.80	24.5	12.50
	34	1.10	-1.50	-0.90	16.8	7.50					
W1	1	-0.30	0.00	-0.20	2.1	0.68	-1.40	-1.20	-0.60	11.2	5.72
	4	-1.60	-0.90	-0.40	5.6	1.81	-0.30	-0.10	0.00	2.1	0.68
	7	-3.30	-1.40	-1.50	22.4	7.30	-1.50	-0.60	-0.20	1.4	0.45
	14	-3.60	-2.00	-1.40	26.6	8.62	-3.40	-1.40	-0.70	23.1	7.50
	21	-3.60	-2.30	-1.30	24.5	7.98	-3.70	-2.20	-1.10	24.5	7.98
	28	-3.20	-2.20	-1.30	14.7	4.77	-3.00	-2.20	-0.90	23.8	7.72
W2	35	-2.90	-1.40	-1.30	8.4	2.79	-3.00	-2.40	-1.40	23.8	7.72
	1	0.00	+0.10	0.00	0.70	0.24	-2.60	-1.60	-1.60	7.0	2.33
	4	-1.10	-0.50	-0.30	4.2	1.46	-0.20	-0.20	-0.10	0.70	0.23
	7	-2.70	-1.40	-1.10	17.5	6.10	-1.60	-0.90	-0.50	5.6	1.86
	14	-2.90	-2.00	-1.20	18.2	6.35	-3.00	-1.40	-0.80	21.0	6.98
	21	-2.80	-1.60	-1.20	14.7	5.13	-3.60	-2.60	-1.10	30.8	10.70
	28	-2.60	-1.50	-1.30	9.1	3.17	-3.10	-2.00	-1.60	14.0	4.65
							-2.60	-2.20	-1.70	9.1	3.02

what closely associated with a recession in chemical activity. Wolbach and Ernst⁴ have shown that young cultures of tubercle bacilli exhibit a certain degree of non-acid-fastness. Neither dextrose, mannite, nor glycerin spare protein to any such degree as dextrose, for example, spares protein under similar conditions with a great majority of the commonly met with bacteria.

Throughout these experiments the growth in plain broth was distinctly less luxuriant and less extensive than that occurring in dextrose,

mannite, or glycerin media. The growths in the later media were of about equal magnitude. After a period of alkalinity the reaction gradually becomes acid in glycerin broth, and progressively alkaline in plain, dextrose, and mannite broths. This is in accord with Theobald Smith's observations mentioned above. The changes in ammonia production would indicate that this development of acid is not to be interpreted as a sparing action of the glycerin for protein. Perhaps the most striking feature of the metabolism is the rather general recession of the

TABLE 1—(Continued)
METABOLISM OF TUBERCLE BACILLI IN BROTH

Bacillus tuberculosis	Days	Mannite Broth					Glycerin Broth				
		Alizarin	Neutral Red	Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
597	4	—0.40	+0.10	—0.10	—2.1	—0.97	+0.50	+0.30	+0.20	0.00	0.00
		—0.30	+0.10	—0.10	—2.1	—0.97	—0.50	+0.10	+0.10	6.3	3.10
	13	—1.90	—0.60	+0.20	21.7	10.00	—1.00	—0.10	+0.40	24.5	12.05
	20	—2.10	—0.90	—0.40	35.2	14.85	—1.30	—0.30	+0.60	35.0	17.25
	27	—2.60	—1.20	—0.60	29.4	15.55	—1.00	—1.10	+0.40	39.9	19.65
W	34	—2.00	—1.40	—0.20	29.4	15.55	—0.80	—0.10	+1.20	44.1	21.70
	4	—0.50	+0.20	—0.10	0.00	0.00	—0.80	+0.10	—0.30	2.1	1.03
	6	—0.70	+0.10	0.00	4.2	1.93	—0.30	+0.20	0.00	2.1	1.03
	13	—3.00	—1.30	—0.80	34.3	15.80	0.00	+0.30	+0.50	19.6	9.68
	20	—2.70	—1.50	—1.00	25.9	11.92	—0.50	+0.20	+0.80	27.3	13.45
W1	27	—2.30	—1.50	—0.90	21.7	10.00	—1.90	—0.10	+0.40	35.7	17.60
	34	—1.60	—1.20	—1.00	13.3	6.13	—0.90	—0.30	+0.40	35.7	17.60
	1	—0.30	—0.30	—0.20	1.4	0.47	—0.20	0.00	—0.30	1.4	0.47
	4	—1.30	—0.40	—0.50	2.1	0.70	—1.50	—0.60	—0.30	5.6	1.86
	7	—2.40	—0.80	—0.40	17.5	5.81	—2.20	—0.80	—0.30	16.8	5.58
W2	14	—3.70	—2.10	—1.20	35.7	11.87	—2.30	—1.20	—0.50	23.8	7.90
	21	—3.30	—3.00	—0.90	34.3	11.40	—2.30	—1.60	+0.10	26.6	8.85
	28	—3.10	—3.20	—1.60	26.6	8.82	—2.70	—1.70	+0.40	27.3	9.08
	35	—3.30	—1.50	—0.90	28.7	9.51	—2.30	—1.30	+0.70	7.7	2.55
	1	—0.30	—0.20	0.00	0.00	0.00	—0.10	—0.10	+0.10	—0.70	—0.24
W2	4	—2.90	—1.40	0.00	7.7	2.62	—1.20	—0.60	—0.10	9.1	3.17
	7	—3.40	—2.20	—1.10	23.8	8.1	—1.30	—1.10	—0.10	18.2	6.34
	14	—4.20	—2.70	—1.60	17.5	5.95	—2.40	—1.90	+0.70	32.2	11.2
	21	—2.70	—2.00	—1.50	14.0	4.76	—1.40	—1.20	+0.10	21.0	7.3
	28	—2.10	—2.10	—1.60	13.3	4.53	—1.80	—1.30	+0.20	21.7	7.53

ammonia. Ammonia production reaches its maximum about the second or third week of incubation, and then gradually diminishes, so that the final amount of ammonia detectable in the cultures is usually much less than that observed at the height of vegetative activity. This phenomenon has not been observed in cultures of other bacteria which have been studied similarly. The reason for this recession is not apparent. Several possible explanations present themselves. Loss of ammonia by volatilization is not very probable, for the solution is not unduly alkaline and the small amount of ammonia formed, taken into con-

sideration with the great solubility of this substance in water, would seem to eliminate this possibility. With our present-day knowledge of autolysis of bacterial cells it would appear that the ammonia should increase rather than diminish if this were the chief cause. In this connection it should be stated that the pellicles showed no visible diminution in size, but this does not exclude the possibility that the contents of the bacteria may have become soluble, leaving their skeletons, as it were, intact.

The fundamental composition of media made from meat juice and peptone must be extremely complex, and it is conceivable that certain substances, perhaps of the nature of fats, lipoids, or their derivatives, might play a part. These substances are present undoubtedly in small amounts in ordinary media. Tubercle bacilli contain considerable amounts of fats and their derivatives in their bodies, and it is conceivable that these substances play some part in this reaction.

CONCLUSIONS

Young, rapidly-growing tubercle bacilli appear to be, in part at least, non-acid-fast.

The strain of avirulent tubercle bacilli studied here exhibit the Theobald Smith reaction characteristic of the growth of human tubercle bacilli in glycerin broth.

Neither dextrose, mannite, nor glycerin appears to exert any marked sparing action for the protein constituents of ordinary media.

Ammonia accumulates rather rapidly during the first, second, and third weeks of growth of tubercle bacilli in plain, dextrose, mannite, and glycerin broths, followed by a definite well-marked recession, during which this ammonia detectable in the media gradually diminishes in amount. The cause of this recession is unknown.

THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

STUDIES IN ACID-FAST BACTERIA. II*

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The study of the metabolism of two rapidly growing, avirulent tubercle bacilli of human origin, in plain, dextrose, mannite, and glycerin nutrient broths, showed a consistent and well-defined progressive breakdown of the protein constituents in all of these media which reached its maximum between the second and the third week (see preceding article). The breakdown of protein was measured by the increase of ammonia. About the third week, ammonia production reached its maximum and then the amount of ammonia, detectable in the media by the method used, showed a definite, progressive recession, so that at the end of five or six weeks the amount of ammonia, altho greater than that in uninoculated controls, was decidedly less than the maximum amount which was found after about three weeks' incubation. No definite explanation for this recession was apparent.

Nutrient broth, as it is usually prepared, contains small amounts of fats, fat derivatives, and lipoids, and it is conceivable that some of these lipoidal substances may play a part in at least the initial phase of this reaction. Lipoids could hardly explain all of the phenomena involved in the ammonia curve, however. Some of these lipoidal substances have attracted considerable attention, particularly in connection with the growth of the tubercle bacillus *in vitro* and in the body. Von Eisler and Laub¹ studied the lipoidal content of the serum of ninety-five tuberculous patients and found it low in all. There was no relation between the decrease of the lipoidal content of the blood and the temperature curve. They found that the cholesterin esters, but not cholesterin itself, were decreased in amount. Deycke and Much² and Sieber

* Received for publication July 27, 1914.

1. Wien. klin. Wchnschr., 1913, 26, p. 968.

2. München. med. Wchnschr., 1909, 56, p. 1986; Berl. klin. Wchnschr., 1910, 47, p. 1933; Centralbl. f. Bakteriöl., Abt. I, Orig. I, 1910, 54, p. 342.

and Metelnikoff³ claim that lecithin, neurin, and cholin dissolve tubercle bacilli in the test-tube, while Löwenstein and Beyer⁴ deny that the lecithin has any bactericidal action on the tubercle bacillus. These latter observers believe that the liberation of acid, incidental to the decomposition of lecithin, is the cause of the destruction of the organisms.

In order to determine what part, if any, these lipoidal substances, in the amounts in which they occur in ordinary media, might play in the metabolism of tubercle bacilli, media were made up from ingredients in which these substances were definitely and quantitatively eliminated. This was accomplished as follows:

Fifteen grams of Fairchild's peptone were extracted for two weeks with ether, two weeks with alcohol, two weeks with acetone, and ten days with petroleum ether, respectively—in a Soxhlet extractor, the successive extrac-

TABLE 1
METABOLISM OF TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

Tubercle bacillus	Days	Plain Broth A					Plain Broth B				
		Alizarin	Neutral Red	Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
597	3	-0.60	-1.00	-0.90	4.2	3.15	-1.40	-1.30	-1.40	11.9	5.32
	7	-1.70	-1.80	-1.30	29.4	22.1	-3.30	-2.80	-1.90	52.5	23.5
	14	-1.90	-1.90	-1.10	38.5	29.0	-3.00	-2.70	-1.70	65.1	29.0
	21	-2.10	-2.10	-1.40	35.0	26.3	-3.80	-3.50	-2.00	60.9	27.2
	28	-1.80	-2.10	-1.10	28.7	21.6	-3.80	-2.90	-1.70	48.3	21.5
W	3	-0.30	-0.50	-0.50	1.4	1.45	-1.40	-1.00	-1.30	11.9	5.3
	7	-1.80	-1.70	-1.30	23.8	17.90	-3.60	-2.90	-1.70	49.7	22.2
	14	-1.60	-1.80	-1.20	37.8	28.4	-2.80	-2.80	-1.80	64.4	28.7
	21	-2.00	-2.20	-1.30	35.0	26.3	-3.70	-3.50	-1.90	60.2	26.9
	28	-1.70	-1.90	-1.30	28.7	21.6	-3.50	-2.80	-1.90	52.5	23.4

tions occurring at intervals of about 12 minutes; these extractions were continued for about six hours daily and for six days per week. Considering the large amount of solvent which bathed this peptone, it is fair to assume that these substances were removed quantitatively. This peptone, which had been extracted, was made into broth by the addition of distilled water: one portion consisting of peptone alone; one portion containing peptone and 1 percent of dextrose; and a third portion containing 3 percent of glycerin in addition to the peptone. Three grams of Na₂HPO₄ and five grams of NaCl per liter were added to each kind of medium. A fourth lot of broth, containing unextracted peptone and the same salts, but with no additional source of carbon, was prepared under the same conditions to serve as a control.

All of the utensils used in the preparation of these fat-free media were cleaned first with alkaline potassium permanganate, then with oxalic acid,

3. Centralbl. f. Bakteriol., Abt. I, Orig., 1910, 54, p. 349.

4. Ibid., 56, p. 160.

then with chromic acid, and washed thoroughly in water and then with distilled water, so that it is certain that no foreign substance could have been introduced into the media during the process of manufacture.

The media thus prepared contained no meat extract or meat juice. It is impossible to rule out the presence of minute traces of dextrose or muscle sugar. These media were distributed in flasks in the usual manner, 100 c.c. per flask, and autoclaved at the same time and inoculated respectively with the two strains of rapidly growing avirulent tubercle bacilli, W. and 597.

The determinations were made in precisely the same manner as those in Study I. The media are designated for purposes of convenience Plain Broth A, Plain Broth B, Dextrose Broth A, and Glycerin Broth A. Plain Broth B, it will be remembered, was made from peptone which had not been extracted. Plain Broth A, Dextrose, and Glycerin Broths were prepared from the peptone which had been extracted as outlined.

Several features in the metabolism of these organisms are noteworthy (Table 1). Plain Broth A (uninoculated) contained 133 mg. of nitrogen per 100 c.c. of broth, while Broth B, which had not been

TABLE 1—(Continued)
METABOLISM OF TUBERCLE BACILLI IN BROTH FREE FROM LIPOIDS AND FATTY SUBSTANCES

Bacillus tuberculosis	Days	Dextrose Broth A					Glycerin Broth A				
		Alizarin	Neutral Red	Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
597	3	—0.70	—0.70	—1.30	6.3	4.50	—0.50	—0.70	—0.60	2.10	1.58
	7	—0.40	—0.40	—1.20	6.3	4.50	0.00	—0.30	—0.60	3.5	2.63
	14	—1.00	—1.00	—1.50	29.4	21.0	0.20	—0.50	—0.80	—0.70	—0.52
	21	—1.50	—1.60	—1.50	23.1	16.5	0.20	—0.60	—0.70	—2.10	—1.58
	28	—1.40	—1.50	—1.40	23.8	17.0	0.20	—0.60	—0.90	—2.80	—2.10
	3	—0.60	—0.70	—1.20	5.6	4.0	—0.30	—0.40	—0.50	3.5	2.63
W	7	—0.60	—0.60	—1.40	1.4	1.0	—0.10	—0.40	—0.80	1.4	1.05
	14	—0.90	—1.50	—1.40	17.5	12.5	0.10	—0.70	—0.80	—2.8	—2.1
	21	—1.50	—1.70	—1.50	23.1	16.5	0.10	—0.60	—0.80	—2.8	—2.1
	28	—1.50	—1.40	—1.40	23.8	17.0	0.10	—0.80	—0.90	—2.8	—2.1

extracted, contained 224 mg. of nitrogen in the same volume. That is to say, during the process of extraction a considerable amount of nitrogenous substance had been removed coincidentally with the removal of the fatty substances. The nature of these nitrogenous substances is unknown. In Plain Broth A and Plain Broth B the ammonia production reaches its maximum at the end of the fourteenth day, at which time it is about 29 percent of the total nitrogen of the media. Notwithstanding the fact that the percentage of ammonia to total nitrogen is the same in both media, the actual amount of ammonia in Plain Broth B, that is, the unextracted broth, is much greater, being, roughly, in the proportion of 65 mg. to 38 mg. It would appear that the

removal of certain nitrogenous substances from Plain Broth A did not materially influence the percentage of protein breakdown as compared with Broth B, and the results suggest, furthermore, that the additional nitrogenous content of Broth B was broken down readily. Plain Broth B was distinctly more alkaline in reaction than Plain Broth A, and this increased alkalinity can be explained tentatively on the basis of the greater production of ammonia in this medium. In the dextrose broth, the amount of ammonia produced was distinctly less than that in the corresponding plain broth. This might be interpreted as a sparing action of the dextrose for the protein constituents of the broth, but this sparing action is far less marked, if indeed it be a sparing action, than is the case with ordinary bacteria studied under the same conditions.⁵ In glycerin broth, after a slight initial increase in ammonia amounting to about 2 percent of the total nitrogen of the medium, the ammonia appears to decrease in amount, so that at the end of the experiments it is less than that contained in the uninoculated media. At the end of the second week, the glycerin broth cultures of both strains of the tubercle bacillus were found to be slightly viscid, and by the end of the fourth week, this viscosity was very marked. The reaction of the medium to phenolphthalein becomes progressively alkaline in spite of this decrease in ammonia. There is no satisfactory explanation for this phenomenon available at the present time. It is conceivable that at least some of this ammonia is tied up in the bodies of the bacteria, and inasmuch as the organisms studied in this connection form firm tenacious pellicles, leaving the medium beneath them perfectly clear and free from bacteria, it was a comparatively simple matter to make a determination of the total nitrogen of the clear underlying broth. The analyses follow.

Organism	Days	Plain Broth A		Plain Broth B		Dextrose Broth A		Glycerin Broth A	
		Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss	Mgs. N per 100 c.c.	Percent Total N Loss
Control	—	133	000	224	000	140	000	133	000
W	28	98	26.3	147	34.3	98	30.0	56	57.9
597	28	77	42.0	154	31.3	96	35.0	42	68.4

5. Kendall, Day and Walker, Jour. Am. Chem. Soc., 1913, 35, p. 1208.

The results show that a very considerable proportion of the total nitrogen in the medium is, apparently, tied up in the bodies of the bacteria. It might be objected at this point that some of this nitrogen may have escaped from the medium, as ammonia, by evaporation, escape perhaps being facilitated by the pellicle floating on the surface. Ammonia, in the amounts produced in these media, almost certainly could not evaporate from the free surface of the medium because of the great affinity of ammonia for water. It is possible, however, that the presence of a pellicle might result in a direct "exhaling" of ammonia into the air, the pellicle acting as a barrier to its reabsorption. This pellicle, it should be remembered, is somewhat dry, and while some loss may have taken place, the amount is probably insignificant when compared with the total amount of nitrogen of the medium. It will be observed that in glycerin broth there was a much greater amount of nitrogen in the pellicle than is the case with the other media. This might be accounted for on the basis of a difference in the luxuriance of the growth, and it is a fact that the pellicles formed, respectively, on Plain Broths A and B are thinner and less extensive than the one formed in glycerin. The pellicle formed in dextrose, however, appears to be quite as dense as that formed in glycerin. It is a noteworthy fact that even the organisms grown in the Plain Broth A, which, theoretically at least, is free from all fats, fatty derivatives, and lipoids, are acid-fast.

While these experiments do not by any means prove that the substance or substances conferring acid-fastness on these organisms are derived from protein derivatives alone, yet it would seem that an experiment of this sort carried out under similar conditions, with especial emphasis on the fat and wax content of the organisms, would throw some definite light on the physiology of the formation of fats and waxes from protein.

The experiments do not explain the recession of ammonia which was noticed in broths containing small amounts of fats and lipoids, and it is probable that these substances do not play any material part in this recession. It is worthy of note that the reaction curve of these organisms in glycerin broth does not conform to the Theobald Smith curve for human tubercle bacilli, the reaction produced being progressively alkaline instead of becoming acid. The composition of the glycerin broth in which these observations were made, however, is so different from that usually employed for this purpose that the results are not at all comparable in the two instances.

THE METABOLISM OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN A MODIFIED USCHINSKY MEDIUM

STUDIES IN ACID-FAST BACTERIA. III*

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In the previous articles the recession of ammonia was observed in old cultures of avirulent, rapidly growing cultures of tubercle bacilli. The cause of this recession was unknown, but probably it is not attributable to small amounts of lipoids or fats.

The present study was made to determine the incidence of this reaction in media of very simple composition. The experiments of Proskauer and Beck,¹ which have since been repeated by many observers,² have shown that the human tubercle bacillus can grow in media of relatively simple composition. These media would seem to offer advantages for the study of the recession of ammonia which we have observed. This advantage lies in the fact that it is possible to provide, as a source of nitrogen, a chemical compound the composition of which is definitely known. For the purpose of this investigation, a medium was made consisting of 4 gm. of asparagin, 2 gm. of di-sodium hydrogen phosphate, and 5 gm. of NaCl to the liter of distilled water, as a basis. This medium was divided into three parts: to one of which was added 1 percent of dextrose; to a second, 1 percent of mannite, and to a third, 3 percent of glycerin, as additional sources of carbon. They were then sterilized under parallel conditions, in 100 c.c. amounts. All of the utensils, which were used in the preparation of these media, were thoroughly freed from all organic matter. It will be seen that the source of nitrogen in this medium is asparagin. The source of carbon is selective, the organisms having a choice of the carbon from the asparagin or from dextrose, mannite, or glycerin, respectively. An attempt was made to grow the organisms in the asparagin solution without any additional source of carbon, but this was unsuccessful.

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1. Ztschr. f. Hyg. u. Infektionskrankh., 1894, 18, p. 128.
2. Frankel, Hyg. Rundschau, 1894, p. 769; Frouin, Compt. rend. Soc. de biol., 1910, 68, p. 915; Lowenstein, Centralbl. f. Bakteriol., Abt. I, Orig., 1913, 68, p. 591; Wherry, Ibid., 70, p. 115; and Jour. Infect. Dis., 1913, 13, p. 144.

TABLE I
METABOLISM OF TUBERCLE BACILLI IN A MODIFIED USCHINSKY MEDIUM

Bacillus tuber- culosis	Days	Dextrose Broth				Mannite Broth				Glycerin Broth						
		Alizarin	Neutral Red	Phenol- phthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenol- phthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenol- phthalein	NH ₃ mgs. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
WI	7															
	14	0.00	+0.20	-0.10	4.9	6.60	-0.30	+0.20	0.00	11.2	12.5	-0.20	+0.10	+0.20	4.2	6.52
	21	-0.10	+0.20	+0.20	4.9	6.60	-0.30	+0.10	+0.20	25.9	39.4	0.00	+0.10	+0.10	4.9	7.61
	28	-0.20	+0.10	+0.40	23.8	32.1	-0.30	-0.10	+0.20	30.8	46.8	-0.10	-0.10	+0.30	9.8	15.2
	35	-0.20	0.00	+0.30	36.4	49.1	-0.50	-0.20	+0.20	39.9	60.3	-0.20	-0.20	+0.30	28.0	43.4
WII	7															
	14	0.00	+0.20	+0.10	4.2	4.55	-0.10	+0.20	+0.10	7.7	8.6	-0.10	+0.20	+0.30	4.2	6.52
	21	-0.10	+0.10	+0.10	6.3	8.49	-0.30	0.00	+0.30	23.1	41.8	-0.10	+0.20	+0.20	4.2	9.78
	28	-0.20	0.00	0.00	14.0	18.9	-0.30	-0.20	+0.20	31.5	48.0	-0.10	-0.10	+0.20	14.0	21.8
	35	-0.20	+0.10	+0.20	36.4	49.1	-0.60	-0.20	+0.10	37.8	57.5	-0.20	-0.10	+0.20	27.3	42.4
S97I	7															
	14	0.00	+0.20	+0.10	3.5	4.87	-0.20	+0.20	+0.10	7.0	7.81	-0.10	+0.10	0.00	2.8	4.35
	21	-0.10	+0.30	-0.10	4.9	6.60	-0.40	+0.10	0.00	21.0	31.9	0.00	+0.10	+0.20	2.1	3.26
	28	-0.10	0.00	+0.50	18.2	24.5	-0.50	-0.20	+0.10	30.1	45.8	-0.10	0.00	+0.20	23.8	37.6
	35	0.00	+0.10	+0.50	32.9	44.3	-0.30	-0.10	+0.10	39.9	60.3	-0.20	-0.10	+0.20	25.2	39.3
S97II	7															
	14	0.00	+0.20	+0.10	30.1	40.5	-0.30	+0.10	0.00	32.2	49.0	-0.10	0.00	+0.20	28.0	43.4
	21	-0.10	+0.00	+0.20	32.2	43.4	-0.30	-0.10	0.00	31.5	48.0	-0.10	0.00	+0.40	31.5	44.7
	28	0.00	+0.10	+0.30	4.2	4.55	-0.20	+0.20	+0.20	7.0	7.81	0.00	0.00	+0.10	2.1	3.26
	35	-0.10	+0.30	0.00	5.6	7.54	-0.40	-0.10	+0.10	21.0	31.9	0.00	0.00	+0.30	2.1	3.26
597II	7															
	14	0.00	+0.20	+0.10	4.2	4.55	-0.20	+0.20	+0.10	7.0	7.81	0.00	0.00	+0.30	2.1	3.26
	21	-0.10	+0.30	0.00	5.6	7.54	-0.40	-0.10	+0.10	21.0	31.9	0.00	0.00	+0.30	2.1	3.26
	28	-0.10	0.00	+0.30	17.5	23.6	-0.30	-0.20	+0.10	30.1	45.8	-0.05	-0.20	+0.30	10.5	16.3
	35	-0.20	+0.10	+0.30	31.5	42.4	-0.40	-0.20	+0.10	39.9	60.3	-0.05	-0.20	+0.30	25.2	39.3
597I	7															
	14	0.00	+0.20	+0.10	31.5	42.4	-0.10	+0.20	+0.10	31.5	48.0	-0.05	-0.10	+0.10	23.8	37.6
	21	-0.10	+0.00	+0.30	31.5	42.4	-0.30	-0.10	+0.20	31.5	48.0	-0.10	-0.10	+0.30	23.2	50.0
	28	-0.10	0.00	+0.30	31.5	42.4	-0.30	-0.10	+0.20	31.5	48.0	-0.10	-0.10	+0.30	23.2	50.0
	42	-0.10	0.00	+0.30	31.5	42.4	-0.30	-0.10	+0.20	31.5	48.0	-0.10	-0.10	+0.30	23.2	50.0

The determinations were made in duplicate at weekly intervals for six weeks, and, with the exception of glycerin, the same recession of ammonia appears, as was noted in the previous experiments. The glycerin does not show this recession. The extent of the recession is somewhat less marked than in peptone-containing media, but the growth, it should be noted parenthetically, was much less luxuriant. Nevertheless, the changes induced in these media, both in reaction and in the decomposition of the nitrogenous constituents, were of sufficient magnitude to warrant the conclusion that the bacteria grew with moderate luxuriance. It was observed previously (Study II) in a medium containing peptone, which had been thoroughly extracted with organic solvents, that the reaction became progressively alkaline in glycerin. In the asparagin medium containing glycerin, the reaction was slightly, but consistently, acid to phenolphthalein. This was also the case in the dextrose and mannite media. The reaction with alizarin as an indicator, on the contrary, was uniformly alkaline. The amount of acid produced, however, was very slight, amounting to from 0.3 to 0.5 c.c. of normal acid per 100 c.c. of medium. Inasmuch as these organisms undoubtedly derive their nitrogen from the nitrogen of the asparagin, it might be assumed that the acid formed was due to the removal of the basic group of the asparagin. There is a certain amount of evidence in favor of this supposition, for the organism in question would not develop in this asparagin solution without an additional source of carbon, either mannite, gelatin, or dextrose, in the experiments cited. At the same time, the breakdown of asparagin, as measured by the increase in ammonia, was so great that it would be unjustifiable to assert that these substances exert a sparing action for the nitrogen. The extent of the breakdown of asparagin was, roughly, the same in each of these media. Altho the maximum degree of nitrogen metabolism was apparently not reached even at the end of six weeks in the glycerin medium, at the end of four weeks, generally speaking, the maximum growth was reached in the other media.

It is a noteworthy fact that, even in these very simple media, the tubercle bacilli, particularly during the last three weeks of growth, were completely acid-fast. During the initial stages of growth, that is, during the first and second weeks, there was a fair proportion of non-acid-fast rods. This is in harmony with the observations made in previous experiments in which more complicated media were used. Morphologically, the organisms were indistinguishable from those

grown on the customary glycerin media, and their staining reactions, when judged by the rather crude methods available at the present time, particularly acid-fastness, were the same as those of tubercle bacilli derived from tuberculous lesions or cultures of tubercle bacilli of maximum virulence on ordinary media. In other words, the tubercle bacillus is able to build up the nitrogenous portion of its substance from asparagin, a relatively simple amino acid derivative, and its acid-fastness, be it a wax or a fat, from substances no more complex than dextrose, mannite, or glycerin, and perhaps some carbon from asparagin.

One of the noteworthy changes produced in the media by the growth of the organisms in the asparagin medium was the development of a mucinous-like substance, which was apparent even at the end of the first week. By the end of the second week it had apparently reached its maximum, altho it persisted throughout the course of the experiment. It was possible to draw out the medium in long and viscid strings by touching it with a platinum needle. Altho the medium underlying the pellicle of the tubercle bacillus exhibited this mucinous change most strongly, the organisms themselves were also somewhat mucinous in character. This viscosity was most marked in mannite, considerable in dextrose, and relatively slight in glycerin.

The relative luxuriance of growth of tubercle bacilli in this very simple medium has somewhat more than academic interest, for it has been shown by various authors that the tubercle bacillus can produce tuberculin from these compounds. Proskauer and Beck¹ showed that their cultures of tubercle bacilli would produce tuberculin in their asparagin medium, and this observation has been confirmed a number of times since. It would appear to be desirable to prepare tuberculin from a simple medium of this type, if possible, because the amount of extraneous substance is very little, consequently the reactions induced by it should be more clean-cut and less liable to misinterpretation, due to the elimination of the irritant action of peptone and other products of protein disintegration which contaminate tuberculin prepared in the usual manner.

The composition of commercial tuberculin varies greatly (White and Hollender³), and it is conceivable that tuberculin developed in a medium of known composition could be standardized much more accurately when it is relatively free from extraneous products than

when it is mixed with substances of unknown composition and of themselves irritant.

After this work was completed, an article by Möllers⁴ appeared, which would seem to indicate that, in an asparagin medium containing glycerin, there is a very considerable antigen content. It is interesting to note that the maximum antigen content coincides, in time, with the greatest weight of the pellicle formed on the surface of the medium, as shown by Möllers⁴ and Lockemann.⁵ After reaching a maximum, both the weight of the pellicle and the antigen content, according to their observations, decrease materially in amount. Möllers concludes that the course of the weight curve of the tubercle bacillus in fluid media shows a great similarity to the curve of the antigen content of the corresponding culture fluid. It was an interesting and significant fact that the greatest weight of the pellicle of the tubercle bacillus reached a maximum and then decreased, suggesting a relationship with the formation of ammonia, which also reaches a maximum and is then followed by a recession of the ammonia content. It is conceivable that the three phenomena, the decrease in weight of the tubercle bacillus pellicle, the decrease in antigen content, and the decrease in vegetative activity, as shown by the ammonia curve, are parallel phenomena, and the cause of the decrease in each instance is closely associated with the recession of this vegetative activity.

4. Möllers. Veröffentlichungen der Robert Koch-Stiftung zur Bekämpfung der Tuberkulose, 1914, 10, p. 56.

5. Lockemann, *Ibid.*, p. 21.

THE METABOLISM OF CERTAIN RAPIDLY GROWING TUBERCLE BACILLI IN MEDIA WITH INOR- GANIC SALTS AS SOURCES OF NITROGEN

STUDIES IN ACID-FAST BACTERIA. IV *

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It is surprising to find that an organism as complex in its activities as the tubercle bacillus should develop in a medium in which the source of nitrogen is an ammonium salt and the source of carbon an alcohol or a carbohydrate. Wherry¹ has shown that a rapidly growing strain of an avirulent tubercle bacillus will develop, with moderate luxuriance, in a medium even simpler in composition than the asparagin medium, referred to in our Study III. Wherry used ammonium chlorid as a source of nitrogen in his experiments, the various simple alcohols and carbohydrates as sources of carbon. It is essential, as he showed, to add phosphorus to these media, preferably Na_2HPO_4 , where ammonium chlorid is used as a source of nitrogen, and also a certain amount of NaCl as well. Even avirulent tubercle bacilli do not grow rapidly on this medium, but the growth is unmistakable and it may be increased somewhat in luxuriance by repeated transfers. The morphology of the tubercle bacillus is considerably modified during prolonged growth on this ammonium chlorid medium, the organisms being for the most part non-acid-fast with a preponderance of coccoid elements, some of them containing acid-fast granules, the majority of the granules, however, being metachromatic.

These observations have been repeatedly confirmed in this laboratory. They are of theoretical importance; in the first place, the organisms developing under these conditions may be regarded as tubercle bacilli reduced in their chemical composition to the lowest terms compatible with growth. They contain, theoretically, but seven elements; viz., carbon, nitrogen, hydrogen, oxygen, phosphorus, sodium, and chlorine. It is conceivable that minute traces of sulphur and perhaps

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1. *Centralbl. f. Bakteriol., Abt. I, Orig.*, 1913, 70, p. 115; *Jour. Infect. Dis.*, 1913, 13, p. 144.

other elements may be absorbed by the media from the air, but these contaminations, if they were present, were reduced to a minimum by thoroughly cleaning the glassware with which they came in contact with alkalin permanganate, oxalic and chromic acids, and water. The media were made from carefully distilled water. The cultures, furthermore, were protected during incubation by double paper caps, and they were grown in an electrically heated incubator. It would appear, therefore, if these precautions were successful, that living tubercle bacilli, containing but seven elements in their body substance, would develop in a medium containing, as a source of nitrogen, an inorganic salt, ammonium chlorid, and a simple alcohol, ethyl alcohol, as a source of carbon. These organisms, furthermore, can exist indefinitely under these conditions, for cultures in this medium have been carried on several months by repeated transfers without a diminution in the extent of the growth; indeed, there is a tendency for the growth to increase somewhat in luxuriance with repeated transfers.

The development, however, in this medium is slow, and more luxuriant growths are obtainable from the same elements using $(\text{NH}_4)_2\text{HPO}_4$ as a combined source of phosphorus and nitrogen, the other constituents remaining the same. The metabolism of a rapidly growing tubercle bacillus (597) in a medium consisting of 4 grams of $(\text{NH}_4)_2\text{HPO}_4$ and 5 grams of NaCl, dissolved in a liter of redistilled water as a basis, to which was added respectively 1 percent of dextrose, 1 percent of mannite, and 3 percent of glycerin as sources of carbon, is presented in this article. The organism was grown for several weeks in this medium, being transferred weekly before the inoculations reported on here were made. It is obvious that this procedure eliminated the possible introduction of small amounts of foreign substances at the time of inoculation and insured successful development of the organism. In experiments involving such simple media, it is obviously essential to exclude extraneous substances by a rigorous attention to the cleaning of all utensils coming in contact with this medium, the details of which have been described. This procedure may be confidently relied on to exclude extraneous contaminations.

It must be remembered that ammonia formation cannot be observed in media in which the source of nitrogen is diammonium hydrogen phosphate, because the ammonia in this compound is removed quantitatively by the Folin air current method in a strongly alkaline solution;²

2. Folin, *Jour. Biol. Chem.*, 1912, **11**, p. 523.

consequently, the successive determinations of nitrogen in this study are, in reality, determinations of the total nitrogen in solution in the media, and the differences observed between the initial nitrogen content and that found at various stages of growth represent the amounts of nitrogen which is locked up in the bodies of the newly formed bacteria, and perhaps also in some substance or substances formed coincidentally by the bacteria which is not detected by this method. If there is such a nitrogen-containing substance or substances, which is not a part of the bacterial cell and which is not detectable by the air current method, it must at least be a synthetic product of bacterial action, for the original nitrogen content can be determined quantitatively by this method.

The tubercle bacillus, according to the analyses of various observers, contains a high percentage of phosphorus amounting to, at least, 50 percent of the ash of the organism,³ and it is conceivable that some combination of phosphorus with simple amino acids or complex combinations of amino acids might be represented in this non-determinable nitrogen fraction. The experiments of Neuberg and Oertel⁴ indicate that the nitrogen of these substances would not escape detection by the air current method, for these substances are labile and easily broken down in acid or alkaline media, and may even decompose on standing.

The results of such a metabolism experiment in this medium are given in Table 1.

The reaction to phenolphthalein in dextrose and mannite is uniformly alkaline; after the first week it becomes progressively acid in glycerin. Inasmuch as the media are fundamentally of the same composition, except for the source of carbon, this experiment would suggest that the organism in question produces acid incidentally to its utilization of glycerin, while it produces alkali when dextrose and mannite are the sources of carbon. This agrees with the observations of Theobald Smith on this point.⁵ The reaction in dextrose is slightly acid to alizarin, in mannite it is neutral, but in glycerin it is slightly alkaline. The entire series of observations on changes in reaction to various indicators, when the same organism is grown in media varying in composition from the relatively complex nutrient broth-peptone medium to the simple ammonium phosphate glycerin medium, would suggest that these reactions are due to unknown constituents or com-

3. De Schweinitz and Dorset, *Jour. Am. Chem. Soc.*, 1898, 20, p. 618; 1903, 25, p. 354; and 20th Annual Report of the Bureau of Animal Industry, 1903.

4. *Biochem. Ztschr.*, 1914, 60, p. 491.

5. *Jour. Med. Research*, 1905, 13, p. 253.

TABLE 1
METABOLISM OF TUBERCLE BACILLUS IN MEDIA WITH INORGANIC SALTS AS SOURCES OF NITROGEN

Days	Dextrose					Mannite					Glycerin			
	Alizarin	Neutral Red	Phenol-phthalein	NH ₃ mg. per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenol-phthalein	NH ₃ mg. per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenol-phthalein	NH ₃ Total N ₂ Percent
7	+0.10	-0.30	-0.10	-3.5	-8.34	-0.10	-0.30	-0.10	-3.5	-8.34	-0.10	-0.10	-0.10	-10.00
14	+0.20	-0.30	-0.10	-4.2	-10.00	-0.20	-0.40	-0.20	-4.2	-10.00	-0.20	0.00	0.00	-10.00
21	+0.30	-0.50	-0.15	-4.2	-10.00	0.00	-0.40	-0.40	-4.2	-10.00	-0.20	+0.10	+0.10	-11.66
28	+0.20	-0.30	-0.05	-2.8	-6.56	0.00	-0.20	-0.20	-2.1	-5.00	-0.30	+0.10	+0.30	-10.00

binations of constituents of the media, and that they are of value for the identification of these organisms only when the composition of the medium is definitely known, or, in the case of media as complex in composition as the glycerin meat juice peptone broth, where the conditions of preparations are rigorously duplicated each time. This is not to be construed, however, as indicating that these reactions under proper conditions are worthless, for, even in the complex nutrient media ordinarily used for the growth of the tubercle bacillus, these reactions appear to be definite and reliable in the hands of those who pay sufficient attention to the details of composition and preparation. The same mucinous substance, which was found abundantly in cultures of the organism grown in the asparagin medium, was found in the ammonium phosphate medium, altho somewhat less in amount. It was most marked in the mannite medium, less in dextrose, and practically absent in glycerin.

THE NITROGENOUS METABOLISM IN AMMONIUM PHOSPHATE MEDIUM

The nitrogen content of this medium was uniformly 42 mg. per 100 c.c. At the end of fourteen days the tubercle bacillus (597) had so acted on the nitrogen constituent that 10 percent of it had disappeared from the underlying culture medium; it is probable that a large percentage of this nitrogen was combined in the bodies of the organisms. A certain amount of evidence in favor of this view is afforded by the reappearance of some of this nitrogen in solution in the dextrose and mannite media on the twenty-eighth day. This experiment would suggest that the cause for the recession of the ammonia was, in some way, associated with the autolysis of the pellicle of the tubercle bacilli, which formed on the surface of the medium. There is no recession in the case of the glycerin broth. This observation is in harmony with similar observations in more complex media, where this recession of ammonia was found to be less marked when glycerin was present than when dextrose or mannite were present.

SUMMARY

A rapidly growing strain of human tubercle bacilli (597) has been grown in a medium of known and very simple composition, consisting essentially of diammonium-hydrogen-phosphate, as a combined source of nitrogen and phosphorus, and dextrose, mannite, and glycerin, respectively, as sources of carbon. At the end of two weeks, 4.2 mg.

of nitrogen, that is to say, 10 percent of the total nitrogen of the uninoculated medium, has been so changed by the growth of this organism that it cannot be recovered as ammonia. This loss of ammonia is most plausibly explained on the assumption that it has been built up into the bodies of the newly developed bacteria. At the end of four weeks, between 40 and 50 percent of this "lost" nitrogen has reappeared in the clear medium underlying the pellicle of the tubercle bacilli in such a form that it can again be determined as ammonia. The period during which the disappearance of nitrogen from the culture fluid is the greatest corresponds with the period of maximum vegetative activity in the culture. Coincidentally with the reappearance of this nitrogen, which can be detected as ammonia in solution, there are evidences of a cessation of vegetative activity. This strongly suggests that the reappearance of this ammonia is associated with a certain amount of autolysis of the bodies of the bacteria.

THE METABOLISM OF "LEPRA BACILLUS," GRASS
BACILLUS, AND SMEGMA BACILLUS IN
PLAIN, DEXTROSE, MANNITE, AND
GLYCERIN BROTHS

STUDIES IN ACID-FAST BACTERIA. V.*

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The study of the metabolism of two avirulent, rapidly growing strains of the human tubercle bacillus in plain, dextrose, mannite, and glycerin broth, respectively, showed two distinct phases in the development of the culture; an initial phase, in which the morphological picture was characterized by the development of a considerable proportion of non-acid-fast bacilli, associated with a progressive increase in the breakdown of the protein constituents of the medium, as shown by the increased formation of ammonia. This initial phase, which reached its maximum at about the third week, was succeeded by a second, recessive phase, in which all of the bacteria were acid-fast, and in which, furthermore, the ammonia which had accumulated during the initial phase gradually decreased, or at least gradually became undetectable by the method used (the Folin air current method), until finally it had practically disappeared. This same phenomenon was observed when these organisms were grown in simpler media; even in a medium as simple in composition as di-ammonium hydrogen phosphate as a combined source of nitrogen and phosphorus, with dextrose as a source of carbon, and NaCl to maintain the proper osmotic pressure of the solution.

The question arises, is this somewhat unusual series of events met with in cultures of other acid-fast bacteria, not tubercle bacilli, grown under the same conditions? If this recession of ammonia is a feature of the growth of the majority, or all acid-fast bacilli in broth, it would appear to differentiate these organisms somewhat sharply from other non-acid-fast bacteria, for the latter organisms either do not exhibit this phenomenon, or exhibit it to a lesser degree.

With this possibility in view, the metabolism of three representative types of the acid-fast group of bacteria, the grass bacillus, the smegma

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TABLE 1
METABOLISM OF *LEPRA BACILLUS* (DUVAL)

Mannite Broth						Glycerin Broth				
Days	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
1	0.00	-0.30	-0.10	+9.8	+3.04	-0.20	-0.10	0.00	1.4	0.44
3	+0.10	-0.20	-0.30	0.00	0.00	+0.20	+0.10	-0.10	1.4	0.44
10	-0.20	-0.30	-0.80	-4.9	-1.52	-0.40	-0.10	-0.50	-0.7	-0.22
21	-0.10	-0.50	-0.90	-4.9	-1.52	-1.80	-1.00	-0.80	-5.6	-1.74
28	-0.40	-0.80	-0.60	-9.8	-3.04	-1.70	-1.10	-1.30	-9.1	-2.82
43	-1.20	-1.80	-1.40	-6.3	-1.95	-1.30	-1.40	-1.30	-11.2	-3.48
51	-1.60	-1.40	-1.70	-13.3	-4.13

TABLE 2
METABOLISM OF *GRASS BACILLUS* III.

Plain Broth						Dextrose Broth				
Days	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
1	-0.10	-0.10	-0.10	-0.7	-0.22	-0.90	-0.20	-0.70	9.8	3.04
3	-0.30	-0.40	-0.20	1.4	0.44	-0.80	-0.30	-0.90	-0.7	-0.22
6	-1.00	-0.40	-1.10	4.2	1.30	-2.20	-0.90	-1.80	-0.7	-0.22
10	-2.70	-1.40	-1.70	14.7	4.57	-2.90	-1.20	-1.60	5.6	1.74
15	-1.90	-2.10	-1.50	24.5	7.60	-3.50	-2.80	-2.00	23.8	7.40
21	-3.60	-4.00	-2.00	23.8	7.40	-3.50	-2.00	-2.20	21.7	6.75
28	-2.50	-2.00	-1.60	20.3	6.30	-3.50	-2.80	-2.20	16.1	5.00
36	-3.10	-1.30	-1.90	7.0	2.2	-2.80	-2.20	-2.30	8.4	2.60
43	-2.20	-2.10	-1.80	1.4	0.44	-2.50	-2.10	-2.40	1.4	0.44
52	-1.90	-2.00	-2.00	-4.9	-1.52	-2.30	-1.80	-2.10	-4.9	-1.52

TABLE 3
METABOLISM OF *SMEGMA BACILLUS*

Mannite Broth						Glycerin Broth				
Days	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
1	-0.30	-0.30	-0.30	8.4	2.60	-0.60	-0.20	-0.30	-0.7	-0.22
3	-1.00	-0.50	-0.70	0.00	0.00	-0.10	0.00	-0.20	-0.7	-0.22
6	-1.70	-0.70	-1.30	0.00	0.00	-1.00	-0.40	-0.50	+4.2	1.30
10	-1.40	-0.80	-0.80	1.4	0.44	+0.10	-0.70	-0.30	-2.8	-0.87
15	-1.30	-1.60	-0.50	16.8	5.22	-0.70	-0.40	-0.50	-2.8	0.87
21	-1.50	-1.20	-0.90	16.8	5.22	+1.20	+1.60	+0.60	+12.6	+3.91
28	-2.90	-2.30	-1.50	28.0	8.70	+0.70	+1.10	+0.60	14.0	4.40
36	-2.20	-2.20	-1.80	14.0	4.35	-0.40	-0.80	-0.60	0.7	0.22
43	-2.40	-2.10	-1.60	19.6	6.04	+0.70	-0.60	0.00	0.7	0.22
52	-1.50	-2.20	-2.00	-5.6	-1.74	-0.30	-0.80	-0.40	-4.9	-1.52

TABLE 1
 METABOLISM OF LEPTA BACILLUS (DUVAL)—(Continued)

Plain Broth						Dextrose Broth				
Days	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
1	0.00	—0.10	0.00	0.00	0.00	—0.70	—0.30	—0.50	—0.7	—0.22
3	+0.10	0.00	—0.20	0.00	0.00	—0.10	0.00	—0.60	—1.4	—0.44
10	—1.30	—0.60	—1.20	0.00	0.00	—1.30	—0.60	—1.20	0.00	0.00
21	—0.10	—0.40	—0.70	0.7	0.22	—2.00	—1.10	—1.00	—1.4	—0.44
28	—2.30	—1.50	—1.50	—2.8	—0.87	—2.00	—1.00	—0.90	—12.6	—3.92
43	—2.30	—1.90	—2.20	—12.6	—3.92	—2.10	—1.50	—2.20	—16.8	—5.20
51	—1.40	—1.40	—1.90	—11.9	—3.70	—1.50	—1.60	—2.20	—14.7	—4.56

 TABLE 2—(Continued)
 METABOLISM OF GRASS BACILLUS III

Mannite Broth						Glycerin Broth				
Days	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
1	+0.10	—0.20	—0.40	9.8	3.04	—0.40	—0.10	—0.10	7.7	2.39
3	+0.10	—0.30	—0.40	0.00	0.00	—0.70	—0.20	—0.30	0.00	0.00
6	—0.10	—0.30	—0.70	0.7	0.22	—1.20	—0.50	—1.00	0.00	0.00
10	—1.10	—0.50	—0.90	1.4	0.44	—0.70	—0.30	—0.40	7.7	2.39
15	—1.50	—1.50	—1.10	8.4	2.60	—0.90	—1.20	—0.30	13.3	4.13
21	—1.50	—1.30	—1.30	15.4	4.78	—0.20	—0.50	—0.30	16.8	5.22
28	—2.10	—1.90	—1.30	11.9	3.70	—1.10	—0.90	+0.30	13.3	4.13
36	—1.90	—1.70	—1.40	16.1	5.00	—0.50	—1.40	+0.10	15.4	4.78
43	—1.80	—1.90	—1.80	2.8	0.88	0.00	—1.20	0.00	4.9	1.52
52	—1.60	—1.70	—1.80	—4.9	—1.52	—0.50	—0.60	—0.20	1.4	0.43

 TABLE 3
 METABOLISM OF SMOGMA BACILLUS—(Continued)

Plain Broth						Dextrose Broth				
Days	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Alizarin	Neutral Red	Phenolphthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent
1	—0.80	—0.40	—0.50	9.1	2.83	—1.50	—0.40	—0.90	8.4	2.61
3	—1.20	—0.50	—1.10	0.7	0.22	—1.20	—0.60	—1.10	—0.7	—0.22
6	—1.90	—1.00	—1.90	5.6	1.74
10	—2.20	—1.30	—1.50	11.2	3.48	—2.90	—1.50	—1.10	18.9	5.87
15	—3.60	—2.20	—1.50	19.6	6.08	—3.00	—2.30	—1.10	22.4	6.96
21	—3.40	—1.20	—1.60	7.7	2.39	—4.10	—1.80	—2.10	28.0	8.70
28	—1.50	—1.60	—1.40	7.7	2.39	—3.50	—2.80	—2.10	20.3	6.30
36	—0.70	—1.20	—1.30	0.00	0.00	—3.90	—2.60	—1.20	23.8	7.40
43	—1.70	—1.80	—1.80	—4.2	—1.30	—3.30	—2.40	—2.40	18.2	5.66
52	—2.00	—2.20	—2.20	—7.7	—2.39

bacillus, and the "lepra bacillus" isolated by Duval, were studied in plain, dextrose, mannite, and glycerin nutrient broths. The technic of experimentation throughout was that used for the study of the tubercle bacillus.

The results, shown in tabular form, are self-explanatory in the light of the observations made on the rapidly growing, human tubercle bacilli. The smegma bacillus and the grass bacillus, altho they do not form as much ammonia under parallel conditions as did the tubercle bacilli, present a well-marked maximum followed by a steady decline in the amount of ammonia detectable in the media in which they were grown. The "lepra bacillus" appears to be somewhat different from the grass and smegma bacilli. First, in that the amount of ammonia produced is very slight, the maximum, 1.40 mg. per 100 c.c. broth, being found in the mannite medium at the end of twenty-four hours' incubation. In all media there was actually less ammonia after a few days' incubation than there was in the uninoculated control. This observation agrees with one recorded previously,¹ where, however, the experiment was only carried on for nine days. The cultural reactions and the curve of metabolism of this bacillus would seem to distinguish it rather sharply from the two organisms mentioned above.

SUMMARY

The metabolism of the smegma and grass bacilli resembles that of the rapidly growing, human tubercle bacilli, described previously, in two important particulars; neither dextrose, mannite, nor glycerin exhibits any appreciable sparing action for the protein constituents of the broth, the amounts of ammonia produced being practically the same in these media as in plain broth; and their cultures present a gradual increase in proteolysis to a maximum which is followed by a clearly defined recession of the metabolism indicated by a gradual decrease in the ammonia content.

The "lepra bacillus" does not present this metabolic phenomenon. This would suggest that this bacillus was entirely distinct in its cultural relationships from the grass and smegma bacilli, which follow more closely the metabolism of the tubercle bacillus.

1. Kendall, Day and Walker, *Jour. Am. Chem. Soc.*, 1913, 35, p. 1248.

THE OCCURRENCE OF A SOLUBLE LIPASE IN BROTH CULTURES OF TUBERCLE BACILLI AND OTHER ACID-FAST BACTERIA

STUDIES IN ACID-FAST BACTERIA. VI*

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The growth of two rapidly growing strains of human tubercle bacilli in media of varied composition showed consistently two distinct phases in their metabolism: an initial phase characterized by rapid vegetative activity and progressive proteolysis, as indicated by a steady increase in ammonia formation; and a second phase marked by a progressive decrease in vegetative activity, and by recessive changes in metabolism, during which the ammonia accumulated during the initial phase gradually disappeared from the culture media (These Studies, I-V).

This second phase, which appears to begin rather regularly after three weeks' growth in ordinary media with the organisms studied, is of unknown causation. It is quite probable that the accumulation of waste products plays a prominent part in restricting the activity of the organisms, but the simple restriction of bacterial growth *per se* does not explain the associated phenomenon of the recession of the ammonia which has been produced during the period of rapid development. Furthermore, attempts to correlate the recession of ammonia with the presence of certain substances in the media, in which these observations were made, were unsuccessful, for even in a culture solution as simple in composition as $(\text{NH}_4)_2\text{HPO}_4$, NaCl, and dextrose, the same phenomenon was observed, suggesting strongly that the explanation of the recession is to be sought for in the organisms themselves. This at once focuses attention on the possibility of ferments, exo- or endo-cellular, being concerned, the entire process, perhaps, being one in which autolysis plays a prominent part.

The tubercle bacillus and other acid-fast bacteria are notoriously resistant to solvents which will promptly destroy other organisms not acid-fast. In the animal body, similarly, these bacteria are resistant to

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lytic powers which usually suffice to destroy corresponding numbers of the ordinary organisms. Fats and waxes, which are present in considerable amount in the substance of these acid-fast bacteria, appear to confer this resistance to solution on these organisms. Consequently, the removal or modification in composition of these substances would appear to be an essential factor in the autolysis of the acid-fast type of bacteria.

Comparatively little appears to have been written on the subject of fat-splitting by bacterial ferments. It is claimed by Rubner¹ and others that the microbic decomposition of fats with the liberation of fatty acids and glycerin is in reality a "fat fermentation" brought about by the direct activity of the protoplasm of the organism, not to a definite fat-splitting ferment elaborated by them. On the other hand, Sommaruga² and others appear to have demonstrated lipases in several types of bacteria; Carrière³ demonstrated a lipase in the bodies of six-months-old tubercle bacilli, but not in the media in which they were grown; Wells and Corper⁴ have also demonstrated lipases in cultures of tubercle bacilli.

In order to determine whether esterases or lipases play a part in the development of the tubercle bacillus in artificial media, a series of cultures of various strains of acid-fast bacilli of various ages, including not only human tubercle bacilli of various degrees of virulence, but also bovine and avian tubercle bacilli as well, and, in addition, certain other acid-fast bacteria, including the grass bacillus, the leprosy bacillus isolated by Duval, and the smegma bacillus, were examined for evidence of ester- and fat-splitting ferments. The organisms investigated, without exception, grew as a firm, tenacious pellicle on the surface of the medium, leaving the underlying fluid perfectly clear. This clear fluid, free from bacteria, was used in these experiments.

The technic adopted was as follows: One cubic centimeter of this clear fluid underlying the bacteria was placed in a large, clean test-tube with 10 c.c. of freshly distilled water, using sterile precautions; 0.25 c.c. of ethyl butyrate (neutral in reaction) and 0.5 c.c. of toluene were then added, and the whole shaken one hundred times in order to produce a well-mixed emulsion.⁵ Two drops of phenolphthalein were then added and the mixture brought to the neutral point by titration with N/50 acid or alkali, depending on the reaction. This mixture was then stoppered and incubated at 37 C. for twenty-four hours, and again brought to neutrality with N/50 alkali. The increase in acid-

1. Arch. f. Hyg., 1900, 38, p. 67.

2. Ztschr. f. Hyg. u. Infektionskrankh., 1890, 18, p. 441.

3. Compt. rend. Soc. biol., 1901, 53, p. 320.

4. Jour. Infect. Dis., 1912, 11, p. 388.

5. Jobling and Bull. Jour. Exp. Med., 1912, 16, p. 483.

ity, measured in terms of N/50 NaOH, was taken as a measure of the lipolytic activity of the culture.

All of the cultures tested for lipase in this series were grown in broth, uniform in composition, in volume (100 c.c.), and with the same area of free surface. It will be seen that the increase in acidity observed represents, theoretically, 1/100 of the total lipolytic activity of the particular culture under discussion, or, better, 1/100 of the amount of acid which would be liberated by the entire culture in twenty-four hours under the given conditions, for it will be remembered that but 1 c.c. of the culture was used for the test: the results, therefore, are comparative, and roughly quantitative.

Almost without exception, the culture media in which these acid-fast bacteria had grown were rather dark-colored, and this color was sufficient in undiluted solutions to obscure somewhat the end point to phenolphthalein during titration. For this reason 1 c.c. of the culture medium was diluted with 10 c.c. of freshly boiled, distilled water. This dilution accomplished three results: boiling the water drove out all CO₂, and the re-entrance of CO₂ was prevented by the layer of toluene which formed a seal on the surface; diluting the broth with 10 c.c. of water also diluted the color proportionately, so that the end point was very much more distinct; finally, the dilution also prevented any considerable concentration of products of ferment activity which might otherwise tend to arrest the process.

It was found by experiment that 0.25 c.c. of ethyl butyrate was sufficient to react with all the lipase present in the media. This ethyl butyrate was freed from acid before it was added to the media.

Toluene was added for two reasons: first, to prevent the absorption of CO₂ from the air and thus increase the acidity of the medium; and, secondly, to restrain the development of any extraneous bacteria which might have inadvertently reached the solution during the process of preparation and titration: 0.5 c.c. of toluene was sufficient to accomplish these two results.

When the diluted broth, ethyl butyrate, and toluene were prepared in the manner indicated above, the tube in which they were placed was agitated thoroughly in order to mix the substances as intimately as possible.

As a rule, the initial reaction of the lipase solution thus prepared was approximately normal to phenolphthalein at the start, but occasionally cultures were found in which there was a slight initial acid or alkaline reaction. In every instance, the reaction was adjusted to a faint pink by the addition of N/50 acid or alkali, as the case might be, prior to incubation.

At the end of twenty-four hours' incubation at 37 C. the tubes were again shaken and the reaction determined by titration with N/50 NaOH. Incubation was continued for seventy-two hours in the early determinations to measure the total ester-splitting power of the media, but the increase in acidity occasionally detectable after twenty-four hours was found to be so slight it was disregarded in these tables, which are designed to show the presence, rather than the exact measure, of the lipase produced by these organisms. Indeed, duplicate determinations on the same sample indicate that the inherent errors of the method (indicator error and error of sampling) practically compensated for these very slight increases which were occasionally detected after seventy-two hours' incubation. The average difference between duplicate determinations is about 0.1 c.c. N/50 NaOH, and this figure appears to represent the average error of the method. The increase in acidity in cubic centimeters of this solution was taken as a measure of the activity of the lipase in the culture medium. It should again be remarked parenthetically that the theoretical lipase activity of the entire culture would be one hundred

times the amount determined in the process described above, for but 1/100 of the medium was used for this determination.

Throughout these experiments, controls were made in the following manner. A media control in which 1 c.c. of uninoculated (sterile) media of the same composition as that of the cultures was prepared in precisely the manner outlined above, the only difference being that the media control was free from any products of bacterial development. A second media control was made in which the broth containing the lipase was incubated under parallel conditions without the ester. This latter control was designed to show any changes in reaction which might take place spontaneously in the medium in the absence of the ester. A third ester control was used in which the ester was prepared with toluene and distilled water, but to which no broth, either inoculated or uninoculated, was added. These controls were incubated under precisely the same conditions as the lipase solutions, and, without exception, these controls have invariably been neutral at the end of twenty-four hours: that is to say, there was absolutely no increase in acidity in these controls which contained uninoculated broth and an ester, inoculated broth and no ester, and an ester alone. Consequently, the accumulation of acid observed in these experiments after twenty-four hours is attributable to the splitting of the ethyl butyrate by a reactive substance developed in these media during the growth of the organisms, resulting in the liberation of butyric acid. From the conditions of the experiment it would appear that this substance, which split the butyric ether, was a fat-splitting ferment. It might be objected that a ferment which splits ethyl butyrate should be termed an esterase and not a lipase: such esterases, which do not decompose complex glycerids, occur in the human body. Achard and Clerc, and Saxl⁶ have shown that an esterase which splits monobutyrim is present in the blood serum of man; they were, however, unable to demonstrate the presence of a true lipase. In order to answer this objection, the same series of experiments was carried out, using castor oil in place of ethyl butyrate as a substrate. The castor oil was first neutralized and emulsified by the cautious addition of N/10 NaOH until a faint permanent pink reaction resulted, according to the method of Kanitz.⁷ This emulsion remained permanent for several days: 0.5 c.c. of this emulsion was added to a series of tubes prepared precisely as those described above, with the same controls.

Culture "597" is a rapidly growing human tubercle bacillus which was obtained from the American Museum of Natural History of New York through the kindness of Professor Winslow. Culture "W" is an avirulent, rapidly growing, human tubercle bacillus which came from the laboratory of Professor Wherry of the University of Cincinnati. Culture "257" is also a human tubercle bacillus intermediate in its growth and virulence between the rapidly growing strains just described and cultures human and human "X," which were obtained from Prof. Theobald Smith of the Harvard University Medical School. These latter cultures, human and human "X," are experimentally pathogenic for guinea-pigs. The bovine tubercle bacillus came originally from the Bureau of Animal Industry, as did the avian culture. For these last two organisms we are indebted to Dr. Enos Day, Director of the Pathological Laboratory at the Chicago Stock Yards. The leprosy bacillus was one isolated by Duval. The smegma bacillus and Grass bacillus III were cultures the histories of which are unknown to us.

6. Achard et Clerc, *Compt. rend Soc. de biol.*, 1902, 54, p. 1144. Saxl, *Biochem. Ztschr.*, 1908, 12, p. 343.

7. *Ztschr. f. physiol. Chem.*, 1905, 46, p. 482.

Table 1 shows the organism, the medium in which the organism was grown, the age of the culture, and the amount of acid in terms of N/50 NaOH, which developed when 1 c.c. of each of these cultures, respectively, was incubated twenty-four hours under the conditions outlined above. It will be observed that, as a general rule, the degree of acidity was somewhat greater when ethyl butyrate was used as a substrate than when castor oil was used for the same purpose, under the same conditions. While the amounts of acid produced by the bacteria shown in this table are not large, it must be remembered that the theoretical acidity, which would be developed by the entire culture under parallel conditions, would have been 100 times the values given. It would appear, therefore, that, without exception, these acid-fast

TABLE 1
AMOUNT OF ACID PRODUCED

Organism	Medium	Age of Culture	C.C. N/50 NaOH	
			Ethyl Butyrate	Castor Oil
B. tuberculosis-Human 597..	Mannite broth	3 months	2.10	1.85
B. tuberculosis-Human W...	Glycerin broth	4 months	1.25	1.15
B. tuberculosis-Human X...	Glycerin broth	4 months	1.35	1.20
B. tuberculosis-Human	Glycerin broth	4 months	1.80	1.65
B. tuberculosis-Human 257..	Glycerin broth	1 month	1.30	1.35
B. tuberculosis-Avian	Glycerin broth	1 month	1.25	1.35
B. tuberculosis-Bovine	Glycerin broth	2 months	1.00	0.95
B. leprae (Duval)	Glycerin broth	1 month	0.35	0.20
B. smegmatis	Glycerin broth	1 month	1.00	0.90
B. grass IV	Glycerin broth	1 month	1.20	1.15
Control (media)	Glycerin broth	—	0.00	0.00
Control (ester)	—	—	0.00	0.00

organisms produce, as the result of their growth in artificial media, a substance which is in solution and which possesses the property of splitting both ethyl butyrate and castor oil. From the nature of this splitting it would appear justifiable to designate this substance a lipase.

In order to throw some light on the physical properties of this lipase, a series of experiments was made with the same organisms, using different cultures, however, to determine the degree of resistance of this lipase to heat. A series of initial experiments was made in which it was found that heating to 60 C. for thirty minutes had little or no effect on the activity of the lipase. A second series of experiments was made in which 1 c.c. of the respective culture fluids, suspended in 10 c.c. of distilled water, was heated at the temperature of boiling water for fifteen minutes, and then, after the addition of the

ester and toluene, compared under precisely the same conditions with unheated cultures of the same bacteria. It will be seen from Table 2 that even heating to 100 C. for fifteen minutes is without noteworthy effect on the activity of this lipase. At first sight, this unexpected resistance of the reactive substance to heat would seem definitely to eliminate a ferment as the cause of this splitting of the ester and castor oil, for the vast majority of ester- and fat-splitting ferments which have been studied up to the present time have been found to be thermolabile, losing their activity at temperatures above 60 C. Carrière found that the lipase of the tubercle bacilli, which he studied, was thermolabile. Loevenhart,⁸ however, has shown that the lipase of the pan-

TABLE 2
EFFECT OF HEAT ON THE LIPASE

Organism	Medium	Age of Culture, Days	C.C. N/50 NaOH Ethyl Butyrate	
			Unheated	Heated
B. tuberculosis W.....	Plain broth	21	1.40	1.35
B. tuberculosis W.....	Dextrose broth	21	1.40	1
B. tuberculosis W.....	Mannite broth	21	1.45	1.25
B. tuberculosis W.....	Glycerin broth	21	1.35	1.30
B. leprae (Duval).....	Plain broth	21	—0.15	—0.25
B. leprae (Duval).....	Dextrose broth	21	—0.20	—0.20
B. leprae (Duval).....	Mannite broth	21	—0.15	—0.20
B. leprae (Duval).....	Glycerin broth	21	—0.10	—0.25
B. smegmatis.....	Plain broth	21	1.20	1.20
B. smegmatis.....	Dextrose broth	21	1.20	0.80
B. smegmatis.....	Mannite broth	21	1.40	1.15
B. smegmatis.....	Glycerin broth	21	1.50	1.20
B. grass IV.....	Plain broth	21	1.30	1.10
B. grass IV.....	Dextrose broth	21	1.25	—
B. grass IV.....	Mannite broth	21	1.10	1.40
B. grass IV.....	Glycerin broth	21	1.20	1.30

creas, when dried, can be heated at 110 C. for one and one-half hours with the loss of about 50 percent of its activity when measured on ethyl butyrate, altho its action on olive oil remained undiminished. Abbott and Gildersleeve⁹ have shown that certain proteolytic enzymes of bacterial origin may be heated in the moist state to a temperature of 100 C. for fifteen to thirty minutes without impairing their characteristic function. Other observers also have found that ferments of bacterial origin may be thermostabile. With these observations in mind it appears logical to attribute the splitting of ethyl butyrate and castor oil observed above to the action of a true lipase.

8. Jour. Biol. Chem., 1907, 2, p. 451.

9. Jour. Med. Research, 1903, 10, p. 61.

Most ferments are said to be non-diffusible. In order to test the diffusibility of the lipase found in the cultures of acid-fast bacilli, two distinct methods were tried: diffusion through agar and diffusion through collodion membranes.

To determine the diffusibility of the lipase through agar, a series of test-tubes of uniform diameter (1.5 cm.) and length (15 cm.) were bent at right angles midway of the long axis, so that the resulting formation resembled a capital "L." Each test-tube was then filled to about two-thirds of its length with glycerin agar, slanting the agar in that portion of the tube nearer the open end, and then inoculating the slanted surface with one of the rapidly growing human tubercle bacilli. In this way a maximum surface growth of the organism was obtained. These cultures were incubated and examined at regular intervals as follows: One of the bent agar tubes was broken in such a manner that the agar in the closed end was removed absolutely free from the growth on the slanted surface, thus eliminating bacteria. This agar was divided into two parts, A and B; A being nearer the slanted surface, B being more remote from the slanted surface. These portions of agar were then macerated with sterile precautions in 5 c.c. of sterile distilled water, 0.25 c.c. of ethyl butyrate added together with 0.5 c.c. of toluene, shaken, neutralized, and the whole then incubated at 37 C. for twenty-four hours. If any lipase had diffused from the bodies of bacteria on the slanted surface of the agar through to the closed arm from which these samples were taken, the customary splitting of the ethyl butyrate and castor oil should take place. A series of twelve such determinations was made, growths from one week to three months being examined in the series, with absolutely uniform negative results; that is to say, this experiment would indicate that an active lipase had not diffused in demonstrable amounts into and through the agar. The organisms grown on the surface, however, contained lipase in every instance. This experiment was not deemed conclusive, for it is conceivable that the composition and reaction of the agar may have inhibited the action of the ferment.

Another attempt was made to determine the diffusibility of the lipase by suspending a collodion sac, 1 cm. in diameter and 10 cm. long, filled with broth known to contain an active lipase, in a test-tube 2 cm. in diameter, the space between the collodion sac and the walls of the tube being occupied by sterile distilled water, with a layer of toluene overlying both the contents of the sac and the water outside the sac.

Even after a week's incubation at 37 C., the fluid outside the collodion membrane being tested daily after addition of ethyl butyrate in the usual manner, no evidence of lipase activity could be detected outside the collodion sac. The fluid within the collodion membrane showed uniformly strong lipolytic action both on ethyl butyrate and castor oil. This experiment was repeated in detail, using the bodies of tubercle bacilli, which had been washed thoroughly and then killed with toluene water, in place of the broth culture containing the lipase; the supposition being that, as the tubercle bacilli autolyzed, a certain amount of lipase might escape from them and diffuse through the collodion membrane into the surrounding distilled water. Here again the results were uniformly negative, altho lipase of considerable strength could be demonstrated in the fluid inside the collodion membrane, where, it will be remembered, the tubercle bacilli were undergoing autolysis.

CONCLUSIONS

The evidence indicates that a variety of acid-fast bacteria, including various strains of the human tubercle bacillus, the bovine, and avian tubercle bacilli, as well as the leprosy, smegma, and grass bacilli, form lipase during their growth in glycerin broth.

The lipase is present in the medium free from the bacteria.

The lipase resists an exposure of fifteen minutes to 100 C. in the moist state without appreciable diminution in its activity.

The lipase formed by these organisms appears to be non-diffusible, at least in an active state, through either agar or a collodion membrane.

THE RELATIVE ACTIVITY OF THE SOLUBLE LIPASE AND LIPASE LIBERATED DURING AUTOLYSIS OF CERTAIN RAPIDLY GROWING TUBER- CLE BACILLI

STUDIES IN ACID-FAST BACTERIA. VII*

A. I. KENDALL, A. W. WALKER AND A. A. DAY

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In the previous article,¹ experiments were recorded which demonstrated that an active fat-splitting ferment was present in solution in broth cultures of a variety of acid-fast bacilli. These experiments were carried out in such a manner as to be roughly quantitative, and the activity of the lipase, as determined by these experiments, was found to be very considerable. It is a matter of importance to determine whether these lipases are excreted by these acid-fast bacilli as exo-ferments, or whether they are bound up in the bodies of the bacteria, and appear in solution only when the bacteria have autolyzed (endo-ferments). Carrière² has described a thermolabile lipase in the organisms of a six-months-old culture of the tubercle bacillus; his observations suggest that this lipase was an endo-ferment and that even minute amounts of culture medium destroy or inactivate it. This observation is not in harmony with the results presented above, for the lipase of the acid-fast bacteria described there was present and active in the culture medium, and it was thermostabile. It is possible, however, that the lipase, which is demonstrable in younger cultures of tubercle bacilli, may have disappeared, as the cultures were incubated for a longer period, without affecting the activity of a second thermolabile endo-lipase which is locked up within the bacteria themselves.

The following observations were undertaken with a view of determining the relative amounts of lipase, respectively, in the culture medium and in the bodies of the bacteria grown in the culture medium. The cultures (various acid-fast bacilli) were of unequal ages, as indicated in Table 1. Various degrees of autolysis of the bacteria, therefore, must have taken place, depending on the strain of organism studied and the length of incubation.

* Received for publication July 27, 1914.

1. Study VI.

2. Compt. rend. Soc. de biol., 1901, 53, p. 320.

The plan of procedure was as follows: For the determination of the lipase in solution in culture media, the method described in the previous article³ was followed exactly. In order to determine the activity of the lipase present in the organisms themselves (which form a tenacious pellicle on the surface of the media), the bacteria were first separated quantitatively from the culture medium and washed free of adherent soluble lipase by filtration through filter paper, then washed three successive times with redistilled water, each time with 100 c.c.

The organisms, freed from all adherent culture medium by this thorough washing, were suspended in 10 c.c. of redistilled, freshly boiled water containing toluene to kill them, then they were mixed intimately with 1 c.c. of ethyl butyrate and 1 c.c. of castor oil, respectively; and again shaken after the further addition of 0.5 c.c. of toluene. A second set of experiments, using parallel cultures of the same bacteria was prepared, using castor oil in place

TABLE 1
AMOUNT OF LIPASE IN MEDIUM AND IN BODIES OF BACTERIA

Organisms	Medium	Age	Cc. N/50 NaOH			
			Filtrate		Bacteria	
			Ethyl Butyrate	Castor Oil	Ethyl Butyrate	Castor Oil
B. tuberculosis W.....	Glycerin	3 months	1.25	1.00	4.05	3.80
B. tuberculosis 597.....	Mannite	2 months	1.10	0.60	2.30	2.10
B. tuberculosis-Human X ..	Glycerin	3 months	1.35	1.20	3.30	2.25
B. tuberculosis-Human	Glycerin	3 months	1.00	0.90	2.30	1.70
B. tuberculosis-Human 257..	Glycerin	1 month	1.30	0.95	3.35	2.55
B. tuberculosis-Avine	Glycerin	1 month	1.60	1.40	3.70	2.90
B. tuberculosis-Bovine	Glycerin	3 months	1.95	1.40	3.55	2.45
B. leprae (Duval)	Glycerin	3 weeks	0.65	—	4.10	—
B. grass III	Glycerin	3 weeks	1.60	—	5.60	—
B. smegmatis	Glycerin	3 weeks	1.55	—	3.55	—
Control.....	Glycerin	—	0.00	0.00	0.00	0.00
Control.....	—	—	0.00	0.00	0.00	0.00

of ethyl butyrate as the substrate. The filtrates of these parallel cultures gave practically identical results, and, for the sake of brevity, but one set of results is reported under "filtrates," as shown in Table 1. The suspensions containing the bacteria were then neutralized to phenolphthalein, using N/50 acid or alkali, as the case demanded. It should be remarked parenthetically that, without exception, the reaction of the organisms after this washing was practically neutral, so that one or, at most, two drops of N/50 acid or alkali were required to bring the reaction to the neutral point of phenolphthalein. The bacteria and the filtrates, prepared according to the procedure outlined, with suitable media and ester controls, were then incubated at 37 C. for twenty-four hours, and the increase in acidity determined by titration with N/50 NaOH. The suspensions of bacteria were further titrated for three successive days, at the end of which time the activity of the lipase was greatly diminished.

Table 1 shows the lipase content, respectively, of the broth and the bacteria in terms of cubic centimeters of N/50 alkali. It will be observed that the determination of lipase in the broth was not carried beyond the twenty-fourth hour of incubation; with the bacteria, however, daily titrations were made, the observations being prolonged for three days. The amount of lipase in the filtrate was found to be between 0.65 and 2.00 c.c. of N/50 acid, but it must be remembered that the total lipase content of the 100 c.c. of the broth, from which the bacteria were derived, would be one hundred times this amount, theoretically. The amount of lipolytic activity exhibited by these organisms, while considerable in amount, is noticeably less than the total theo-

TABLE 2
AMOUNT OF LIPASE IN WASHINGS AND IN BACTERIA AUTOLYZED AFTER WASHING

Organisms	Medium	Age	Cc. N/50 NaOH to Neutralize Ethyl Butyrate							
			24 Hours Incubation				Bacteria Autolyzed after Washing			
			Filtrate from Bacteria	Washings			Incubation			
				A	B	C	24 Hrs.	48 Hrs.	72 Hrs.	Total
B. tuberculosis W	Plain broth	6 weeks	0.65	0.05	0.00	0.00	2.35	2.30	0.30	4.95
B. tuberculosis W	Dextrose broth	6 weeks	1.30	0.10	0.00	0.00	2.80	2.90	0.75	6.45
B. tuberculosis W	Mannite broth	6 weeks	1.15	0.05	0.00	0.00	2.35	2.20	0.20	4.75
B. tuberculosis W	Glycerin broth	6 weeks	1.75	0.05	0.00	0.00	2.30	1.60	0.30	4.20
B. tuberculosis 597	Plain broth	6 weeks	0.45	0.05	0.00	0.00	2.20	2.15	0.50	4.85
B. tuberculosis 597	Dextrose broth	6 weeks	1.20	0.10	0.00	0.00	2.85	3.30	0.15	6.30
B. tuberculosis 597	Glycerin broth	6 weeks	1.70	0.20	0.00	0.00	3.15	2.25	0.60	6.00
Control	Medium and ester	—	0.00
Control	Washings and ester	0.00	0.00	0.00
Control	Ester	0.00	0.00

retical fat-splitting power of the underlying culture medium. The cultures had been incubated for the most part from one to three months, so that it would be justifiable to assume considerable autolysis before these determinations were made. Nevertheless, the very considerable disparity observed between the relatively large amount of lipolytic activity of the filtrates on the one hand, and of the bacteria which grew in these culture media on the other, would suggest the possibility that the ferment in the culture medium, in part at least, was an extracellular lipase, unless it be assumed that this lipase was an endo-ferment which had escaped from the substance of the bacteria into the underlying medium during their autolysis prior to these experiments. The experiment does not determine this point.

In order to determine the amount of lipase lost in washing these bacteria and to throw some light on the possible influence of the source of carbon on the production of lipase, an experiment was made as follows: two rapidly growing human tubercle bacilli, "W" and "597," were grown for six weeks in plain, dextrose, mannite, and glycerin broths, respectively. At the end of that time the organisms were separated quantitatively from the underlying medium by filtration through filter paper, then they were washed with three successive portions of distilled water. The washings were labelled A, B, and C, respectively. The determinations were made as indicated in Table 2.

The results indicate that the filtrates from plain broth cultures were uniformly less active lipolytically than the filtrates of the same organisms grown in dextrose, mannite, and glycerin broths. There was very little lipase in the first washing (A), none in the second and third washings (B and C). The results otherwise are self-explanatory.

CONCLUSIONS

The observations appear to justify the conclusion that certain acid-fast bacteria grown in nutrient broth, with dextrose, mannite, and glycerin as additional sources of carbon, produce an active lipase which appears in solution in the various culture media.

The bodies of the bacteria, freed from adherent culture media and soluble lipase by thorough washing, also contain an active lipase, probably liberated as the bacteria underwent autolysis.

The lipase in solution appears to be either greater in amount, or more active than that contained in the bacteria freed from the culture medium.

It cannot be stated whether the lipase free in the culture media is freed as the result of autolysis of the bacterial cells (endo-lipase), or whether it is secreted by the bacteria as an exo-lipase.

OBSERVATIONS ON THE SPECIFICITY AND THERMO-
STABILITY OF THE LIPASE DEVELOPED DURING
THE GROWTH OF A RAPIDLY GROWING
TUBERCLE BACILLUS IN MEDIA OF
VARIED COMPOSITION

STUDIES IN ACID-FAST BACTERIA. VIII *

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The constant presence of a thermostabile lipase, both in the filtrates of broth cultures of certain acid-fast bacilli (including human, bovine, and avian tubercle bacilli, and others) and in the organisms themselves,¹ focuses attention on the possible identity of the lipase produced by these various bacteria. Before this question can be answered, it is desirable to study the effect of the composition of the medium on the nature and extent of the lipolytic activity manifested by a specific organism; for it is conceivable that this lipase may be present in all media in which the given organism will grow, irrespective of composition, or, on the other hand, it may be developed only in the presence of certain substances contained in these media, presumably those on which it can act.

In the first instance, the lipase would be an integral factor in the life history of the organism, whereas in the second case the production of a lipase might be regarded as a definite response on the part of the bacteria to the presence of a specific substance, or substances, in the substrata on which the organisms have grown. If the first assumption is correct, lipolytic activity should be demonstrable even in the simplest media compatible with growth of the organisms; such ferment activity, however, would not necessarily rule out the latter possibility.

The experiments here recorded were designed to show the effect of the composition of the medium on the nature and extent of lipolytic activity exhibited by a rapidly growing human tubercle bacillus (597). The media selected for this purpose varied in composition from one of extreme simplicity, containing ammonium chlorid as a source of nitrogen, and ethyl alcohol as a source of carbon, through others of various

* Received for publication July 27, 1914.

1. See preceding articles.

degrees of complexity to the regulation nutrient broth commonly used for the culture of bacteria, nutrient meat-juice-peptone broth. The qualitative composition of these media is clearly indicated in Table 1, where the sources of nitrogen and carbon are specifically set forth. The composition of the nutrient broths, except for the addition of various carbohydrates, is omitted for obvious reasons. The organism was grown for several successive transfers in each of these media before the lipolytic activity was tested, both to acclimatize it to the various ingredients and to insure maximum growth. The final determinations recorded below were made uniformly after twenty-one days' incubation at 37 C. The technic has been described previously.

Throughout this work controls have been made in the following manner:

Media Controls.—One cubic centimeter of the sterile uninoculated medium was mixed with 10 c.c. of sterile distilled water, and, after the introduction of the ester and toluene, incubated under parallel conditions with the corresponding lipase determinations in the same medium.

Ester Controls.—The appropriate amounts of ester and toluene suspended in 10 c.c. of water were likewise inoculated at 37 C. with the media controls and the lipase solutions. In order to save unnecessary complications in the table, it can be stated definitely that, without exception, both the media controls and the ester controls showed no change in reaction after incubating twenty-four hours in the manner indicated. That is to say, whatever changes are noticed in the lipase-containing solutions are due to the action of the ferment and not to any reactionary changes attributable either to the culture medium itself or in the esters.

Vigorous attention to the cleanliness of all apparatus coming in contact with the culture media has been observed. The organism was grown for several successive transfers in the medium in which the observations were subsequently made. This is a point of considerable importance, for the procedure eliminates the possibility of transfer of active substance at the time of inoculation.

With this explanation, the results shown in Table 1 are self-explanatory. The figures in the table represent the cubic centimeters of N/50 NaOH required to neutralize the acid liberated from the various esters after twenty-four hours' incubation with 1 c.c. of the clear medium underlying the pellicle of bacteria, which forms as the organisms grow. The total lipase activity of each culture is, therefore, theoretically, one hundred times the amount indicated in the table, for it will be remembered that but 1 c.c. of the culture medium is used in these determinations, while the total volume of culture in which the organisms have grown is 100 c.c. in each case. Generally speaking, the results show that the extent of the lipolytic activity increases with

TABLE 1
LIPOLYTIC ACTIVITY OF TUBERCLE BACILLUS 597

Days of Growth	Composition of Media		Cubic Centimeter of N/50 NaOH to Neutralize
	Source of Nitrogen	Source of Carbon	
21	NH ₄ Cl	CH ₃ CH ₂ OH
21	NH ₄ Cl	Glycerin
21	NH ₄ Cl	Dextrose
21	NH ₄ Cl	Mannite
21	Glucose	Mannite
21	(NH ₄) ₂ HPO ₄	CH ₃ CH ₂ OH
21	(NH ₄) ₂ HPO ₄	CH ₃ COONa
21	(NH ₄) ₂ HPO ₄	Glycerin
21	(NH ₄) ₂ HPO ₄	Dextrose
21	(NH ₄) ₂ HPO ₄	Mannite
21	(NH ₄) ₂ HPO ₄	CH ₃ CH ₂ OH
21	Asparagin	Glycerin
21	Asparagin	Dextrose
21	Asparagin	Mannite
21	Asparagin	CH ₃ CH ₂ OH
21	Asparagin (NH ₄) ₂ HPO ₄	Glycerin
21	Asparagin (NH ₄) ₂ HPO ₄	Dextrose
21	Asparagin (NH ₄) ₂ HPO ₄	Mannite
21	Asparagin (NH ₄) ₂ HPO ₄	Plan
21	Nutrient broth	Glycerin
21	Nutrient broth	Dextrose
21	Nutrient broth	Mannite
21	Nutrient broth
21	Control
21		NH ₂ HPO ₄ ,NaCl
21		NH ₂ HPO ₄ ,NaCl
21		NH ₂ HPO ₄ ,NaCl
21		NH ₂ HPO ₄ ,NaCl
21		NH ₂ HPO ₄ ,NaCl
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increased complexity in composition of the medium in which the organism is grown. The lipolytic activity, observed in media in which ammonia, either as ammonium chlorid or diammonium hydrogen phosphate, is a source of nitrogen, is distinctly less than that observed in nutrient broth where amino-acids, and probably other nitrogen-containing bodies, are possible sources of nitrogen. The source of carbon appears to make but little difference in the extent of lipolytic activity, at least in the simpler media where this relationship can be definitely observed. The relative luxuriance of growth of the tubercle bacillus in the various media presented above leads one to believe that the extent and thickness of the pellicle, that is, the luxuriance of the growth of the organism, varies almost directly with the increasing complexity in

TABLE 2
EFFECT OF HEATING LIPASE TO 100 C. FOR 15 MINUTES

Days of Growth	Source of Nitrogen	Source of Carbon	Other Ingredients	Unheated Filtrate Cubic Centimeters of N/50 NaOH					Heated Filtrate (100 C.-15') Cubic Centimeter of N/50 NaOH				
				Ethyl Acetate	Ethyl Butyrate	Amyl Acetate	Amyl Butyrate	Amyl Valerianate	Ethyl Acetate	Ethyl Butyrate	Amyl Acetate	Amyl Butyrate	Amyl Valerianate
22	(NH ₄) ₂ HPO ₄	Sod. Lactate	NaCl	0.55	0.60	0.60	0.45	0.50	0.60	0.50	0.35	0.45	0.50
22	(NH ₄) ₂ HPO ₄	Glycerin	NaCl	0.40	0.50	0.70	0.40	0.50	0.45	0.40	0.80	0.30	0.60
22	(NH ₄) ₂ HPO ₄	Dextrose	NaCl	0.46	0.35	0.40	0.65	0.45	0.25	0.50	0.70	0.35	0.45
22	(NH ₄) ₂ HPO ₄	Mannite	NaCl	0.30	0.35	0.40	0.65	0.55	0.25	0.45	0.50	0.45	0.60

composition of the medium. Therefore, the conclusion that the extent of lipolytic activity varies almost directly with the relative luxuriance of growth appears to be justified. On the other hand, the action of the lipase is about the same on the various esters tested, irrespective of the composition of the medium. This observation is in harmony with the extensive observations of Loevenhart,² who has shown that the same lipase can act on a variety of esters.

It is apparent from the results that the lipase produced by the tubercle bacillus can act on various esters, even tho it is developed by the organisms grown in media of the simplest composition. It should be again emphasized that the organisms were repeatedly trans-

2. Jour. Biol. Chem., 1907, 2, p. 456.

ferred in these simple media before the results presented here were obtained, so that there is practically no possibility that a transfer of lipase, other than that developed by the organisms in the medium under question, can have taken place at the time of inoculation.

Table 2 shows the effect of heating the lipase solution to 100 C. for fifteen minutes before adding the ester and toluene. The results indicate that the lipase demonstrable in the simplest media is thermostabile, agreeing in this respect with the thermostability of the lipase found in the more complex nutrient broth. This is another argument in favor of the identity of the lipase developed in these various media.

CONCLUSIONS

A rapidly growing strain of the human tubercle bacillus produces a lipase which appears to be qualitatively the same when it is grown in media varying in composition from one consisting essentially of ammonium chlorid, ethyl alcohol, Na_2HPO_4 , and NaCl , to the extremely complex nutrient meat-juice-peptone broth ordinarily used for bacterial cultures.

The lipase observed in the simplest media acts on various esters, irrespective of the nature of the carbon compound of the medium in which it is developed. For example, the lipase developed in the $(\text{NH}_4)_2\text{HPO}_4$ mannite medium acts on triacetin and castor oil as energetically as on ethyl butyrate, or other simple esters. That is to say, the lipase developed in the simplest medium acts even on a complex glycerid.

This lipase appears to be thermostabile whether it is tested in the simplest media or in the most complex.

The activity of the lipase appears to be roughly proportionate to the relative luxuriance of the growth of the tubercle bacillus.

A COMPARISON OF THE CURVES OF LIPOLYTIC ACTIVITY AND PROTEOLYSIS OF CERTAIN RAPIDLY GROWING HUMAN TUBERCLE BACILLI IN MEDIA OF VARIED COMPOSITION
STUDIES IN ACID-FAST BACTERIA. IX*

A. I. KENDALL, A. W. WALKER, AND A. A. DAY

(From the Department of Bacteriology, Northwestern University Medical School, Chicago.)

In the previous articles it has been shown that broth cultures of various acid-fast bacteria exhibit lipolytic activity; that this lipolytic activity is also demonstrable in the autolyzed bacteria from the same media; and that the lipase in solution in the broth and in the bodies of the bacteria grown in this broth is thermostabile. Furthermore, this lipase, irrespective of the composition of the medium in which it is produced, acts on various esters and glycerids. The extent of lipase activity observed, both in culture media and in autolyzed bacteria taken from these media, appears to vary with the luxuriance of the growth of the organisms. This observation, however, appears to apply more strictly to the filtered broth cultures than to the autolyzed bacteria obtained from these cultures, and the question arises: Is this lipolytic activity proportionate to the amount of autolysis which the bacteria undergo in the media, or does this lipolytic activity vary with the vegetative activity (metabolism) of the organisms? The solution of this question possesses more than academic interest, for it is conceivable that an active exolipase might play a not unimportant part in the development of the tubercle bacillus in the human body.

If the former possibility alone were realized, tubercle bacilli should exhibit lipolytic activity more or less proportionate to their autolysis; whereas, if the latter possibility alone (or associated with the former possibility) were involved, there would be a rough parallelism between the amount of lipolysis demonstrable in culture and the extent of vegetative activity of the organisms themselves in the same culture, provided appropriate media were used. That is to say, if the tubercle bacillus produces an exolipase, it is probable that the curves of lipolytic activity and vegetative activity would reach their maxima more or less synchronously.

* Received for publication July 27, 1914.

TABLE 1
METABOLISM OF TUBERCLE BACILLI IN MEDIA A, B, AND C

Medium	Days	Dextrose				Mannite				Glycerin			
		Reaction Phenol-phthalein	NH ₄ g. Increase per 100 c.c. Media	NH ₃ Total N Per cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH ₄ g. Increase per 100 c.c. Media	NH ₃ Total N Per cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH ₄ g. Increase per 100 c.c. Media
A	14	-0.10	-4.2	-10.0	0.25	-0.20	-4.2	-10.0	0.30	0.00	-4.2
	21	-0.15	-4.2	-10.0	0.40	-0.40	-4.2	-10.0	0.55	+0.10	-4.9
	28	-0.05	-2.8	6.56	0.35	0.25	-0.20	-2.1	-5.0	0.35	0.30	+0.30	-4.2
B	14	-0.05	+4.9	9.0	0.20	-0.20	+16.1	29.5	0.25	0.05	0.00	+4.2
	21	-0.05	+13.3	24.4	0.50	0.10	-0.20	+20.3	37.2	0.30	0.05	+0.10	+7.0
	28	-0.05	+23.8	43.5	0.75	0.15	0.00	+21.0	38.4	0.65	0.20	+0.10	+12.6
C	14	-0.20	+9.1	10.2	0.45	0.00	+23.1	25.8	0.60	0.05	-0.50	+4.2
	21	-0.20	+18.9	21.1	0.70	0.20	0.00	+23.1	25.8	1.00	0.60	-0.30	+14.0
	28	0.00	+16.1	18.0	0.45	0.20	-0.20	+26.6	29.7	0.90	0.45	+0.10	+17.5

* Figures expressed as NH₃ per 100 c.c. (Medium A) represent the total soluble nitrogen of the clear fluid underlying the bacterial growth: full details in text.

TABLE 2
GROWTH OF TUBERCLE BACILLI IN MEDIUM D

Bacillus Tuberculosis	Days	Dextrose				Mannite				Glycerin			
		Reaction Phenol-phthalein	NH ₄ g. Increase per 100 c.c. Media	NH ₃ Total N Per cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH ₄ g. Increase per 100 c.c. Media	NH ₃ Total N Per cent	Ethyl-butyrate c.c. N/50 NaOH	Castor Oil c.c. N/50 NaOH	Reaction Phenol-phthalein	NH ₄ g. Increase per 100 c.c. Media
WI	21	+0.40	23.8	32.1	0.50	0.65	+0.20	30.8	46.8	0.60	0.55	+0.30	9.8
	28	+0.30	36.4	49.1	0.70	0.65	+0.20	39.9	60.3	0.95	0.65	+0.30	28.0
	35	+0.20	30.8	41.5	0.45	0.15	+0.30	30.8	46.8	0.70	0.15	0.00	36.8
	42	+0.10	34.3	46.3	0.50	0.15	+0.20	31.5	48.0	0.75	0.10	+0.10	33.6
WII	21	0.00	14.0	18.9	0.50	0.55	+0.20	31.5	48.0	0.70	0.55	+0.20	14.0
	28	+0.20	36.0	49.1	0.65	0.65	+0.10	37.8	57.5	0.95	0.65	+0.20	27.3
	35	+0.30	31.5	42.4	0.60	0.30	0.00	30.8	46.8	0.70	0.10	+0.10	32.2
	42	+0.30	32.6	45.3	0.50	0.10	+0.10	33.6	51.1	0.60	0.10	+0.10	35.0
597I	21	+0.50	18.2	24.5	0.50	0.55	+0.10	30.1	45.8	0.60	0.55	+0.20	23.8
	28	+0.50	32.9	44.3	0.75	0.40	+0.10	39.9	60.3	1.10	0.50	+0.20	25.2
	35	+0.50	30.1	40.5	0.45	0.25	+0.10	32.2	49.0	1.45	0.20	+0.20	28.0
	42	+0.29	32.2	43.4	0.40	0.25	0.00	31.5	48.0	0.55	0.20	+0.40	31.5
597II	21	+0.30	17.5	23.6	0.50	0.55	+0.10	30.1	45.8	0.70	0.55	+0.30	10.5
	28	+0.30	31.5	42.4	0.75	0.55	+0.10	39.9	60.3	1.05	0.65	+0.30	25.3
	35	+0.30	31.5	42.4	0.45	0.20	+0.10	31.5	48.0	0.25	0.20	+0.10	23.8
	42	+0.30	31.5	42.4	0.60	0.15	+0.20	31.5	48.0	0.65	0.15	+0.30	32.2

With these two possibilities in view, the following experiments were undertaken to demonstrate the relationship, if such exists, between the lipolytic activity of cultures of certain acid-fast organisms and the metabolism of these organisms, as measured by ammonia formation and a change in reaction. In the present communication the metabolism of two rapidly growing human tubercle bacilli is considered. The procedures followed are precisely those described in previous communications where the full details are given.

Briefly, the metabolism of the tubercle bacilli (vegetative activity) was measured by the changes in ammonia content of the medium, which indicates the action of the organisms on the protein constituents, and the changes in

TABLE 3
GROWTH OF TUBERCLE BACILLI IN MEDIUM E

Bacillus Tuber- culosis	Days	Plain Broth (a)				Plain Broth (b)			
		Phenol- phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol- phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH
597	3	—0.50	1.4	1.45	0.15	—1.30	11.9	5.30	0.20
	7	—1.30	23.8	17.9	0.10	—1.70	49.7	22.2	0.05
	14	—1.20	37.8	28.4	0.10	—1.80	64.4	28.7	0.40
	21	—1.30	35.0	26.3	0.15	—1.90	60.2	26.9	0.15
	28	—1.30	28.7	21.6	0.30	—1.90	52.5	23.4	0.45
W	3	—0.90	4.2	3.15	0.10	—1.40	11.9	5.32	0.20
	7	—1.30	29.4	22.1	0.10	—1.90	52.5	23.5	0.05
	14	—1.10	38.5	29.0	0.15	—1.70	65.1	29.0	0.45
	21	—1.40	35.0	26.5	0.10	—2.00	60.9	27.2	0.45
	28	—1.10	28.7	21.6	0.35	—1.70	43.8	21.5	0.50

reaction to phenolphthalein. The lipase activity was measured according to the method described previously. It consisted, essentially, in suspending 1 c.c. of the bacteria-free filtrate of the various cultures in freshly boiled, distilled water, adding 0.25 c.c. of ethylbutyrate and 0.5 c.c. of toluene, and incubating at 37 C. together with appropriate controls. The increase in acidity in terms of N/50 NaOH is taken as a measure of the lipolytic activity of the culture for the period of incubation mentioned.

The organisms have been studied in media of extremely simple composition, and through successive degrees of complexity to ordinary nutrient broths with various sources of carbon, as follows:

Medium A (Table 1).—(NH₄)₂ HPO₄, 4 gm., and NaCl, 5 gm., in 1,000 c.c. redistilled water with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

It will be observed that the determination "Ammonia" (Table 1) is in reality the determination of the total soluble nitrogen in this medium, for all

the nitrogen of $(\text{NH}_4)_2\text{HPO}_4$ is measured by the Folin Air Current Method of ammonia estimation. The decrease in "ammonia," therefore, observed during the second and third weeks of incubation, represents the amount of nitrogen appropriated by the bacteria as they increased in numbers. The reappearance of nitrogen, observed at the end of the fourth week in the filtrates of the cultures in this medium (associated with a decrease in lipolytic activity of the solution), is probably to be regarded as an indication of autolysis of the bacteria with the liberation from them of nitrogenous substances which again pass into solution, and are recovered as "ammonia."

Medium B (Table 1).—Asparagin, 4 gm., Na_2HPO_4 , 2 gm., and NaCl, 5 gm., in redistilled water, 1,000 c.c., with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

Medium C (Table 1).—Asparagin, 2 gm., $(\text{NH}_4)_2\text{HPO}_4$, 2 gm., Na_2HPO_4 , 1 gm., NaCl, 5 gm. in 1,000 c.c. of redistilled water.

TABLE 3—(Continued)
GROWTH OF TUBERCLE BACILLI IN MEDIUM E

Bacillus Tuber- culosis	Days	Dextrose Broth (a)				Glycerin Broth (b)			
		Phenol- phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol- phthalein	NH_3 mg. Increase per 100 c.c. Broth	NH_3 Total N_2 Percent	Ethyl- butyrate c.c. N/50 NaOH
597	3	—1.20	5.6	4.00	0.15	—0.50	3.5	2.63	0.10
	7	—1.40	1.40	1.00	0.10	—0.80	1.4	1.05	0.15
	14	—1.40	17.5	12.50	0.10	—0.80	—2.8	—2.1	0.15
	21	—1.50	23.1	16.50	0.15	—0.80	—2.8	—2.1	0.20
	28	—1.40	23.8	17.0	0.50	—0.90	—2.8	—2.1	0.10
W	3	—1.30	6.3	4.50	0.15	—0.60	2.1	1.58	0.15
	7	—1.20	6.3	4.50	0.30	—0.60	3.5	2.63	0.10
	14	—1.50	29.4	21.0	0.95	—0.80	—0.70	—0.52	0.15
	21	—1.50	23.1	16.5	0.95	—0.70	—2.1	—1.58	0.15
	28	—1.40	23.8	17.0	0.90	—0.90	—2.8	—2.1	0.10

Medium D (Table 2).—Asparagin, 4 gm., $(\text{NH}_4)_2\text{HPO}_4$, 2 gm., NaCl, 5 gm. in 1,000 c.c. redistilled water.

Medium E (Table 3).—Fairchild's peptone (extracted with ether, alcohol, acetone, and petroleum ether to remove lipoids and fat in Medium E[a] and unextracted in Medium E[b]), 5 gm., Na_2HPO_4 , 2 gm., NaCl, 5 gm. in 1,000 c.c. redistilled water, with 1 percent dextrose, or 3 percent glycerin.

Medium F (Table 4).—Regulation sugar-free meat-juice-peptone broth with 1 percent dextrose, 1 percent mannite, or 3 percent glycerin.

As shown in Table 1, in the ammonium phosphate medium (A), the maximum nitrogen metabolism was reached on the twenty-first day, at which time the organisms had removed 10 percent of the total medium from solution; this nitrogen was probably incorporated in their bodies. Lipase activity is also maximum at this time. By the end of the twenty-eighth day, autolysis of the bacteria was well under

way (shown by the reappearance of some of the nitrogen in solution) and lipolytic activity had diminished somewhat.

In the asparagin medium (B) there is steady increase in metabolism in the dextrose, mannite, and glycerin modifications, respectively, associated with a progressive increase in lipase activity. This experiment was not carried far enough to show the recession in metabolism, but other studies with the same media indicate that at the end of the fourth week there is usually a recession of metabolism.

In the ammonium phosphate-asparagin medium (C) the maximum of metabolism and lipolytic activities is reached on the twenty-first day, except in the glycerin modification.

TABLE 4
GROWTH OF TUBERCLE BACILLUS W IN MEDIUM F

Days	Plain Broth				Dextrose Broth			
	Phenol- phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol- phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH
1	-0.10	0.00	0.00	0.15	-0.50	-0.70	-0.22	0.15
3	-0.30	0.70	0.22	0.20	-1.10	-2.80	0.88	0.55
6	-0.90	2.8	0.88	1.90	-1.60	1.40	0.44	0.75
10	-1.40	16.8	5.22	1.60	-1.60	14.70	4.57	2.50
15	-1.70	22.4	6.96	1.65	-2.10	28.7	8.60	1.75
21	-1.70	26.6	8.30	1.90	-1.80	34.3	10.65	2.60
28	-1.10	14.0	4.40	1.40	-1.70	26.6	8.30	1.40
36	-2.10	5.6	1.74	1.25	-2.40	9.8	3.04	1.05
43	-1.60	0.00	0.00	1.20	-2.50	7.0	2.17	1.10
51	-1.50	-2.8	-0.87	1.15	-2.10	-5.6	-1.74	1.00

The general parallelism between metabolism and lipolytic activity (Medium D) is clearly set forth in Table 2.

Medium E (a) consists of Fairchild's peptone extracted to remove all fats and lipoids. Growth was fairly luxuriant, but lipase activity is surprisingly low (Table 3). No explanation for this disparity can be advanced; certain other peculiarities—the production of a progressively alkaline reaction in glycerin and unusual products of growth—suggest that this peptone medium is acted on differently by the tubercle bacillus than the regulation meat-juice peptone media.

Medium F consists of regulation meat-juice-peptone broth, plain and with dextrose, mannite, or glycerin. The observations (Table 4) have been continued for fifty-one days; the maximal proteolytic activity is reached by the third week, at which time lipolytic activity appears

to be at its height. There is a great diminution in the ammonia content of the media after this time; at the end of fifty-one days' incubation there is actually less than at the start. The lipase activity declines considerably after three weeks, but not proportionately to the ammonia.

The recession of ammonia appears to be associated with autolysis of the bacteria, and in this connection the experiments of Lockemann¹ and Möllers² are suggestive. Lockemann showed that the weight of tubercle bacilli grown in glycerin broth increased steadily to a maximum, then diminished somewhat; and Möllers' observations would indicate that the antigenic content of the same culture paralleled the weight curve of Lockemann. It is a striking coincidence to compare

TABLE 4—(Continued)
GROWTH OF TUBERCLE BACILLUS W IN MEDIUM F

Days	Mannite Broth				Glycerin Broth			
	Phenol- phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol- phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH
1	—0.20	0.00	0.00	0.00	0.00	—1.4	—0.44	0.05
3	—0.40	0.70	0.22	0.40	—0.30	1.4	0.44	0.30
6	—0.80	6.3	1.95	1.85	—0.70	4.9	1.21	2.05
10	—0.70	16.8	5.22	2.20	—0.60	8.4	2.61	2.45
15	—0.80	28.7	8.90	1.80	—0.70	18.2	5.65	2.30
21	—0.70	29.4	9.12	1.65	—0.30	18.9	5.87	3.80
28	—0.60	25.9	8.05	1.45	+0.10	18.9	5.87	1.35
36	—0.60	25.2	7.83	1.30	—0.30	16.8	5.22	1.15
43	—0.50	21.7	6.74	1.35	0.00	11.2	3.48	1.25
51	—0.50	8.4	2.60	1.40	—0.20	—0.70	—0.21	1.15

these curves with the similar curves of metabolic and lipolytic activity, and the possibility suggests itself that these phenomena are closely related, if not identical, in origin.

The lipase curve, following the curve of proteolysis to its maximum, but diminishing far less rapidly during the period of recession, speaks strongly in favor of the view advanced in a previous article, that the lipase is an exoferment, in part at least. Otherwise lipase activity should increase progressively with autolysis, unless its activity is inhibited by the accumulation of products of its own production.

It was a point of some interest to determine just how much nitrogen is contained in the bacterial cells at the end of fifty-one days' incuba-

1. Veröffentlichungen der Robert Koch, Stiftung zur Bekämpfung der Tuberculose, 1914, 10, p. 21.

2. Ibid., p. 56.

tion. This was readily accomplished by comparing the total soluble nitrogen in media after fifty-one days' growth with the initial total nitrogen of media of the same composition inoculated under parallel conditions. The tabulated results show clearly that between 21 and 32 percent of the initial nitrogen is not in solution, and the logical inference is that this "lost" nitrogen is retained in the bodies of the bacteria.

Bacillus Tuberculosis W	Plain	Dextrose	Mannite	Glycerin
Initial total N ₂ -mg.	322	322	322	322
Final total N ₂ -mg.	231	217	224	252
Loss total N ₂ -mg.	91	105	98	70
Percent loss*	28.3	32.6	30.4	21.7

*The percentage of nitrogen not in solution, but incorporated in the bodies of the bacteria which had grown in 51 days.

There is a noteworthy parallelism between the curve of vegetative activity, as measured by ammonia formation, and the curve of lipolytic activity, as measured by the changes in acidity in all media. The period of greatest ammonia production, which is assumed to mark the period of maximum vegetative activity, coincides definitely with the period of greatest lipolytic activity; and the recession of ammonia, which has been commented on in the previous communications, appears to be associated with a decrease in the lipolytic activity of the cultures.

CONCLUSIONS

The period of maximum vegetative activity of broth cultures of certain avirulent, rapidly growing tubercle bacilli, as measured by ammonia formation (proteolysis), appears to coincide with the period of maximum lipolytic content of these cultures, as measured by their action on ethylbutyrate.

Both ammonia production and lipolytic activity are extremely slight during the first day's growth of these organisms, and increase, roughly, proportionately to their respective maxima.

There is a noteworthy recession of both factors after this maximum is reached.

These experiments appear to warrant the assumption that the organisms studied excrete a soluble, active lipase during the period of active development; for if autolysis alone were responsible for the lipolytic activity observed in these cultures, it should increase as autolysis proceeds.

A COMPARISON OF THE CURVES OF LIPOLYTIC ACTIVITY AND PROTEOLYSIS OF CERTAIN ACID-FAST BACILLI IN NUTRIENT BROTHS
STUDIES IN ACID-FAST BACTERIA. X*

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The demonstration of an active lipase in solution in broth cultures of certain rapidly growing human tubercle bacilli, the curve of lipolytic activity of which, measured in terms of liberation of acid from ethyl-butyrate, follows rather closely their curve of protein metabolism,¹ suggests that this lipase may play a part in the preparation of certain nutritional substances for assimilation by these bacteria. There is a certain amount of presumptive evidence in favor of this view, for the lipase appears to be active even after the organisms are removed from the media in which it is developed, and it is most abundant or most active when bacterial development is intense. Furthermore, this lipase acts on a variety of esters and glycerids,² which fact, altho not proving its exogenous functions, suggests that it is sufficiently versatile in its attack to split those substances of a fatty nature it might be confronted with. If this lipase is indeed a true exoferment, it might be justifiable to assume that other bacteria of the same type as the tubercle bacillus (other acid bacteria) would also elaborate such a ferment.

The observations recorded here indicate that filtrates of broth cultures of certain acid-fast bacilli, other than the human tubercle bacillus, do exhibit such lipolytic activity.

The organisms studied in this connection were the so-called leprosy bacillus (Duval), the grass bacillus, and the smegma bacillus. The first organism is of unknown history; the last two were received from Professor Winslow of the American Museum of Natural History. The technical details of measuring the lipolytic activity of such cultures has been described in detail previously,³ and will not be repeated here.

* Received for publication July 27, 1914.

1. See preceding article.

2. Study VIII.

3. Study VI.

TABLE 1
LIPOLYTIC ACTIVITY OF *SMEGMA BACILLUS*

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH
1	—0.50	9.1	2.83	0.00	—0.90	8.4	2.61	0.10
3	—1.10	0.7	0.22	0.15	—1.10	7.0	2.2	0.60
6	—1.90	5.6	1.74	1.15
10	—1.50	11.2	3.48	0.60	—1.10	18.9	5.87	1.75
15	—1.50	19.6	6.08	1.30	—1.10	22.4	6.96	1.75
21	—1.60	7.7	2.39	0.80	—2.10	28.0	8.70	1.95
28	—1.40	7.7	2.39	0.50	—2.10	20.3	6.30	1.00
36	—1.30	0.00	0.00	0.50	—1.20	23.8	7.40	1.10
43	—1.80	4.2	1.30	0.60	—2.40	18.2	5.66	1.60
52	—2.20	—7.7	—2.39	1.30

TABLE 2
LIPOLYTIC ACTIVITY OF *GRASS BACILLUS* III

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH
1	—0.10	0.7	0.22	0.05	—0.70	9.8	3.04	0.20
3	—0.20	1.4	0.44	0.20	—0.90	0.7	0.22	0.40
6	—1.10	4.2	1.30	0.55	—1.80	0.7	0.22	0.75
10	—1.70	14.7	4.57	0.60	—1.60	5.6	1.74	1.40
15	—1.50	24.5	7.60	1.60	—2.00	23.8	7.40	1.75
21	—2.00	28.3	7.40	1.80	—2.20	21.7	6.75	1.65
28	—1.60	20.3	6.30	0.90	—2.20	16.1	5.00	0.80
36	—1.90	7.0	2.20	1.00	—2.30	8.4	2.60	1.05
43	—1.80	1.4	0.44	1.10	—2.40	1.4	0.44	1.15
52	—2.00	—4.9	—1.52	1.30	—2.10	—4.9	—1.52	1.15

TABLE 3
LIPOLYTIC ACTIVITY OF *DUVAL'S LEPROSY BACILLUS*

Days	Plain Broth				Dextrose Broth			
	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl- butyrate c.c. N/50 NaOH
1	0.00	0.00	0.00	0.05	—0.50	—0.7	—0.22	0.00
3	—0.20	0.00	0.00	0.05	—0.60	—1.4	—0.44	0.10
10	—1.20	0.00	0.00	0.05	—1.20	0.00	0.00	0.00
21	—0.70	0.70	0.22	0.20	—1.00	—1.4	—0.44	0.00
28	—1.50	—2.8	—0.87	—0.90	—12.6	—3.92	0.20
43	—2.20	—12.6	—3.92	0.20	—2.20	—16.8	—5.20	0.10
52	—1.90	—11.9	—3.70	0.10	—2.20	—14.7	—4.56	0.10

LIPOLYTIC AND PROTEOLYTIC ACTIVITY OF ACID-FAST BACILLI 469

TABLE 1—(Continued)
LIPOLYTIC ACTIVITY OF *SMEGMA BACILLUS*

Mannite Broth					Glycerin Broth			
Days	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.30	8.4	2.60	—0.05	—0.30	—0.7	—0.22	0.05
3	—0.70	0.00	0.00	0.05	—0.20	—0.7	—0.22	0.05
6	—1.30	0.00	0.00	0.10	—0.50	4.2	1.30	0.95
10	—0.80	1.4	0.44	1.75	—0.50	—2.8	—0.87	1.10
15	—0.50	16.8	5.22	1.20	—0.30	2.8	0.87	1.10
21	—0.90	16.8	5.22	1.60	—0.60	12.6	3.91	1.55
28	—1.50	2.80	8.70	1.10	—0.60	14.0	4.40	1.80
36	—1.80	14.0	4.35	1.00	—0.60	0.7	0.22	1.05
43	—1.60	19.6	6.04	1.65	0.00	0.7	0.22	1.75
52	—2.00	—5.6	—1.74	1.30	—0.40	—4.9	—1.52	1.40

TABLE 2—(Continued)
LIPOLYTIC ACTIVITY OF *GRASS BACILLUS* III

Mannite Broth					Glycerin Broth			
Days	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.40	9.8	3.04	0.10	—0.10	7.7	2.39	0.20
3	—0.40	0.00	0.00	0.15	—0.30	0.00	0.00	0.25
6	—0.70	0.7	0.22	0.75	—1.00	0.00	0.00	0.05
10	—0.90	1.4	0.44	1.60	—0.40	7.7	2.39	1.00
15	—1.10	8.4	2.60	1.80	—0.30	13.3	4.13	1.70
21	—1.30	15.4	4.78	1.80	—0.30	13.3	4.13	1.60
28	—1.30	11.9	3.70	1.30	—0.30	16.8	5.22	1.20
36	—1.40	16.1	5.00	1.40	0.10	15.4	4.78	1.10
43	—1.80	2.8	0.88	1.60	0.00	4.9	1.52	1.05
52	—1.80	—4.9	—1.52	1.30	—0.20	1.4	4.30	1.25

TABLE 3—(Continued)
LIPOLYTIC ACTIVITY OF *DUVAL'S LEPROSY BACILLUS*

Mannite Broth					Glycerin Broth			
Days	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl-butyrate c.c. N/50 NaOH	Phenol-phthalein	NH ₃ mg. Increase per 100 c.c. Broth	NH ₃ Total N ₂ Percent	Ethyl-butyrate c.c. N/50 NaOH
1	—0.10	9.8	+3.04	0.00	0.00	1.4	0.44	0.10
3	—0.30	0.00	0.00	0.05	—0.10	1.4	0.44	0.10
10	—0.80	—4.9	—1.52	0.00	—0.50	—0.7	—0.22	0.05
21	—0.90	—4.9	—1.52	0.00	—0.80	—5.6	—1.74	0.05
28	—0.60	—9.8	—3.04	—1.30	—9.1	—2.82
43	—1.40	—6.3	—1.95	0.05	—1.30	—11.2	—3.48	0.10
52	—1.70	—13.3	—4.13	0.10

The tables indicate that, with the exception of the Duval organism, broth cultures of these acid-fast bacteria do exhibit lipolytic activity; furthermore, there is a tendency for the maximum period of proteolysis to coincide with the period of maximum lipolytic activity. The organisms, however, present certain peculiarities in their metabolism curves which deserve further consideration before their exact significance is set forth, and for this reason only the most general statements are warranted.

TABLE 4

THE INITIAL TOTAL NITROGEN AND THE RESIDUAL TOTAL NITROGEN OF VARIOUS MEDIA AFTER THE ORGANISMS HAD GROWN IN THEM FOR 52 DAYS

	Smegma Bacillus	Lepra Bacillus	Grass Bacillus
Plain broth—			
Initial total N ₂ —mg.	—	322	322
Final total N ₂ —mg.	—	266	231
Loss total N ₂ —mg.	—	56	91
*Percent loss.....	—	17.4	28.3
Dextrose broth—			
Initial total N ₂ —mg.	322	322	322
Final total N ₂ —mg.	224	266	231
Loss total N ₂ —mg.	98	56	91
*Percent loss.....	30.4	17.4	28.3
Mannite broth—			
Initial total N ₂ —mg.	322	—	322
Final total N ₂ —mg.	238	—	245
Loss total N ₂ —mg.	84	—	77
*Percent loss.....	26.1	—	23.9
Glycerin broth—			
Initial total N ₂ —mg.	322	322	322
Final total N ₂ —mg.	301	112	266
Loss total N ₂ —mg.	21	210	56
*Percent loss.....	6.51	6.52	17.4

* Percent loss = percentage of initial total nitrogen which has been appropriated into the bacteria as they have increased in the medium. This nitrogen fraction is subject to variations, increasing as growth increases, and decreasing as vegetative activity wanes and autolytic processes cause partial solution of the bacterial cells.

The Duval bacillus reacts quite differently from the others; altho it grew luxuriantly in all the media, the lipase activity demonstrable in culture appears to be practically nil. Further observations with this organism are in progress.

An approximate measure of the luxuriance of growth of these bacteria, at any stage of the history of the culture, may be obtained by determining the amount of nitrogen which has been tied up in their bodies incidental to their growth. This is readily accomplished by comparing the total nitrogen of the uninoculated medium with that of the clear medium underlying the firm, tenacious pellicle of bacteria which have grown in media of the same composition. Table 4 shows the initial total nitrogen and the residual total nitrogen in milligrams

per 100 c.c. of various media after the organisms had grown in them for fifty-two days. (These bacteria were grown in media of 100 c.c. volume.)

In general, with the exception of the "lepra bacillus," the acid-fast bacteria discussed show a parallelism in their curves of lipolytic activity, as measured by the liberation of acid from ethylbutyrate and their curves of proteolysis. This parallelism is discernible in cultures in plain, dextrose, mannite, and glycerin broths.

THE EFFECT OF CHOLESTEROL ON PHAGOCYTOSIS *

KAETHE DEWEY AND FRANK NUZUM

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Cholesterol appears to be not only an essential constituent of the living cell and to play an important part in the construction of the organs, but also to be a factor in some of the reactions of immunity. In this latter capacity it is, to all appearances, the opposite to lecithin.

Cholesterol prevents the hemolytic action of such toxins as saponin, agaricin, tetanolysin, and vibriolysin. Windaus' method of separating cholesterol from its esters and other combinations is based on its property of forming compounds with toxins, and in particular with digitonin. The neutralizing action on toxins for erythrocytes is attributed to definite atoms in the molecule, according to Hausmann,¹ Abderhalden and LeCount,² and Walbum,³ chiefly to the hydroxyl group. Schreiber and Lenard⁴ tested the comparative antihemolytic action of cholesterol and oxysterol and observed that the latter prevents hemolysis only in higher concentration and in this respect is greatly inferior to cholesterol. This behavior changes as soon as oxysterol is added to cholesterol; the mixture has a stronger action than cholesterol alone in the same amount. Even the addition of a small quantity of oxysterol has this effect. The authors believe that this is due to the fact that the addition of oxysterol to cholesterol gives a better emulsion of the latter.

The relation of cholesterol to the reaction in the Wassermann test has been discussed by several writers. Pighini⁵ found cholesterol in the spinal fluid in cases of dementia praecox and in epilepsy; the serum of these patients also contained abnormal amounts of cholesterol. According to him, this substance may be the chief active constituent in the Wassermann reaction. Walbum³ believes that the action of cholesterol as antigen in this reaction is due to the free hydroxyl group. Gaucher, Paris, and Desmoulière⁶ found an excess of cholesterol in the blood of old syphilitics to be the rule. Walker and Swift state that the antigen value of the extracts for the Wassermann reaction can be increased by the addition of cholesterol. According to Bürger and Beumer,⁷ however, the outcome of the reaction is not dependent on the presence of simply more or less of cholesterol or lecithin.

Walbum,³ Stuber,⁸ and Arkin⁹ studied the effect of cholesterol on phagocytosis. The experiments by Arkin with reference to this ques-

* Received for publication August 14, 1914.

1. Beitr. z. chem. Physiol. u. Path., 1905, 6, p. 567.

2. Ztschr. f. exper. Path. u. Ther., 1905, 2, p. 199.

3. Ztschr. f. Immunität, 1910, 7, p. 544.

4. Bioch. Ztschr., 1913, 54, p. 291.

5. Riforma medica, 1909, 25, p. 20.

6. Bull. acad. de Méd., 1912, 78, p. 55.

7. Berl. klin. Wchnschr., 1913, 3, p. 112.

8. Bioch. Ztschr., 1913, 51, p. 211; and 1913, 53, p. 493.

9. Jour. of Infect. Dis., 1913, 13, p. 408.

tion are but one in a series of tests of various pharmacological drugs for their action on phagocytosis, and were limited to observations in vitro. Walbum made his study in vitro and in vivo. In the former, cholesterol and a number of its derivatives were tested; of these, cholesterol itself and the benzoate and proprionate of cholesterol gave the highest figures. The experiments in vivo were carried out on three rabbits which received intravenous injections of cholesterol in doses of from 0.002 gm. to 0.0002 gm. per kilo of rabbit weight. The blood was taken before and several times after the injection and the serum examined for its opsonic strength with the colon bacillus. Stuber also studied the question by experiments both in vitro and in vivo. In the latter, cats were injected with doses of 30, 25, and 20 c.c. of a 0.5-1 percent suspension of cholesterol. The organism used was the thrush fungus. In both sets of experiments, the action of lecithin was also tested, chiefly with reference to its effect on the action of cholesterol.

The results have not been uniform. In Walbum's tests in vitro the figures of the absolute phagocytosis with cholesterol and its derivatives are all above normal phagocytosis, and those of cholesterol and the benzoate and proprionate are three times as much. From his experiments in vivo the author reports that phagocytosis was doubled and that this action seemed to remain unchanged for a long time. He concluded that since the cholesterol action sets in at once, it might perhaps be of interest to test this method therapeutically. It seemed to him, however, that an increased amount of cholesterol did not alter the action; he states that his experiments were too limited for any definite conclusions. Stuber's results are in striking contrast. The experiments in vitro did not vary in results notably from those in vivo, which were carried out on cats; there was a decided fall in phagocytosis, amounting to from 40 to 70 percent. Lecithin neutralized the inhibiting action of cholesterol; when lecithin is heated for fifteen minutes at 70 C. the neutralizing action is abolished. The action of lecithin on cholesterol was of short duration.

Arkin also obtained a diminished phagocytosis in vitro.

In view of the marked difference in the results of Walbum and of Stuber, it seemed worth while to make a further study of cholesterol with reference to phagocytosis. The object was to determine the opsonic power of the serum of guinea-pigs injected intraperitoneally with increasing doses of cholesterol and that of an animal immunized

with streptococci and simultaneously injected with cholesterol in the same doses as used with the non-immunized animal.

The experiments were continued for twenty days. Thirteen injections were made, nine with an average interval of thirty-six hours, the last four in daily succession. Merck's pure cholesterol was used in a colloidal suspension prepared by the method of Porges and Naubauer,¹⁰ which was employed also by Walbum and Stuber. A 1 percent suspension in doses

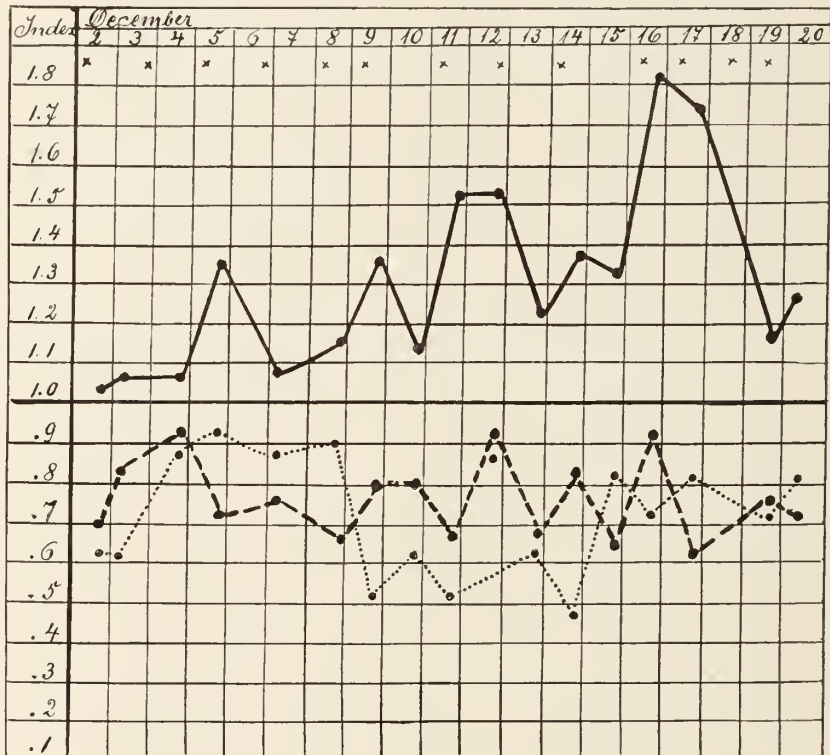


Chart 1.—Curves of opsonic index.

Heavy solid line = guinea-pig immunized against streptococci.

Dotted line = guinea-pig injected with cholesterol.

Heavy broken line = guinea-pig injected with streptococci and cholesterol.

The crosses give the time of the injections of cholesterol.

from 0.1 to 0.4 c.c. was given. Suspensions of killed streptococci in doses from 100,000,000 to 400,000,000 were given in nine successive injections, which were followed by four injections with living streptococci in doses of from 100,000,000 to 300,000,000. A normal guinea-pig and one injected with strepto-

10. Biochem. Ztschr., 1907, 7, p. 152.

cocci served as control animals. Blood was taken from the animals once, and a few times twice, during the period between two injections; human blood furnished the white corpuscles; the organisms for phagocytosis were streptococci. The serum from each animal was mixed with an equal amount of washed human leukocytes and of a streptococci suspension and incubated for fifteen minutes at 37 C. Smears were stained with Leishman's stain. With a few exceptions, 100 cells were counted; the figures of the normal control animal were taken as the unit for the phagocytic ratio. The opsonic curves on Chart 1 illustrate the results obtained.

We see that by introducing small quantities of cholesterol into the systems of guinea-pigs, and thus keeping up a constant supply of this substance in the circulation, there is, in the action of the serum of such animals on washed normal human leukocytes, a reduction of phagocytosis as compared with the action of normal serum of guinea-pigs. This diminution is from 20 to 30 percent. The simultaneous injection of immunizing doses of killed streptococci with the cholesterol results in an opsonic curve which is almost identical with that of serum containing only cholesterol. In these experiments only the serum was tested. These results from the injections of cholesterol are in marked contrast to those reported by Walbum and agree to a large extent with those of Stuber, tho our figures of the reduced index are not as low as his. Since his methods deviate from mine and were chosen with a different object in view, it seemed of interest to study the question with the same methods.

Stuber wanted to study the effect of cholesterol on the leukocytes; one-half hour after the intravenous injection of cholesterol into cats, in quantities which are large in comparison to those used by Walbum and ourselves, 0.03 c.c. of blood was mixed with 0.03 c.c. of a suspension of thrush fungus and 0.1 c.c. of a saturated solution of ovalbumen, (or 0.1 c.c. of artificial serum or 0.1 c.c. of the serum of the same species of animal). This mixture was incubated for one hour at 37 C. and smears were made of the leukocytes which were separated from the mixture by a short centrifugation; 200 leukocytes were counted. Stuber observed that if the leukocytes were washed their phagocytic function was affected; he abstained from this customary method. He obtained a decrease in phagocytosis by 40 to 70 and even 90 percent, and concluded that the action of cholesterol is on the leukocytes themselves, their vital functions being paralyzed. He is not inclined to consider the opsonins of the serum to have the significance which Wright attaches to them. "The conception of opsonins," he writes, "is only the relatively comprehensible expression of possibilities of biological reactions between lipoids of the cell and those of the serum, so that we may conceive of a certain lipoid tonus, to which the cell, bathed in serum, is subjected, and whose visible biologic effect on the leukocytes is manifested by phagocytosis." Stuber found the injurious effect of cholesterol on the vital functions of the leukocytes to be of long duration, as on the sixth day after the injection there was still a reduction of phagocytosis of 50 percent. The

accuracy of Stuber's methods seems reliable, but those to whom opsonins have a practical meaning may question whether his interpretation of his results is the only one possible; the objection might be raised that in his tests of phagocytic power the possibility that the cholesterol acted on the opsonins cannot be excluded.

In the study of this question the following method was used. The opsonic index of three rabbits was tested several times before the injections were made; the corpuscles of each animal were used in succession and no variations in the phagocytic ratio were found beyond those within the limit of error in the technic. Spontaneous phagocytosis was tested also. The index obtained the day before the injection is a fair average of these counts: Rabbit A, 1,320 gm., 1.28; Rabbit B, 1,390 gm., 1.30; Rabbit C, 1,420 gm., 1.20; while spontaneous phagocytosis was 0.28.

Rabbit A was used as control animal No. 1; Rabbit B was injected intraperitoneally with 0.6 c.c. of a freshly prepared 0.5 percent suspension of cholesterol; Rabbit C, was control animal No. 2 and received a similar dose as Rabbit B without the cholesterol, namely, distilled water to which acetone had been added and evaporated. One hour, forty-eight, and seventy-two hours after the injection, the blood was taken from each animal for the serum and for the leukocytes. Coagulation was prevented with citrate solution; the bacterium employed was the staphylococcus, and preparations were stained with carbolthionin.

The results show a definite and constant, tho not very marked, reduction of phagocytosis with normal serum on leukocytes acted on by cholesterol and with serum containing cholesterol on normal leuko-

TABLE 1
EFFECT OF A SMALL DOSE OF CHOLESTEROL ON THE LEUKOCYTES

Time of Taking Blood for Serum and Leukocytes	Leukocytes	Serum	Phagocytosis (Absolute Number of Bacteria per Cell)	Ratio
1 hour after the injection	Rabbit A, Control 1	Rabbit A	2.02	1.00
		Rabbit B	1.48	0.72
		Rabbit C	2.05	1.02
	Rabbit B injected with 0.6 c.c. 0.5 percent suspension of cholesterol	Rabbit A	1.72	1.00
		Rabbit B	0.01	0.35
		Rabbit C	1.76	1.02
	Rabbit C, Control 2; injected with 0.6 c.c. distilled water.	Rabbit A	1.93	1.00
		Rabbit B	1.44	0.74
		Rabbit C	2.00	1.03
72 hours after the injection	Rabbit A, Control 1	Rabbit A	1.54	1.00
		Rabbit B	1.22	0.79
		Rabbit C	1.50	0.98
	Rabbit B, injected with cholesterol	Rabbit A	1.28	1.00
		Rabbit B	0.88	0.69
		Rabbit C	1.20	0.99
	Rabbit C, Control 2, injected with distilled water	Rabbit A	1.56	1.00
		Rabbit B	1.28	0.82
		Rabbit C	1.54	0.99

TABLE 2

COMPARISON OF THE ACTION OF SERUM AND OF THE LEUKOCYTES IN PHAGOCYTOSIS AFTER THE INJECTION OF A SMALL DOSE OF CHOLESTEROL

Time of Taking Blood	Normal Serum on Normal Leukocytes	Normal Serum on Leukocytes of Animal Injected with Cholesterol	Serum of Animal Injected with Cholesterol on Normal Leukocytes	Serum of Animal Injected with Cholesterol on Its Own Leukocytes
1 hour after the injection	2.02	1.72	1.48	0.61
	2.05			
	1.93	1.76	1.44	
	2.00			
Ratio	1.00	0.86	0.73	0.35
72 hours after the injection	1.54	1.28	1.22	0.88
	1.50			
	1.56	1.20	1.28	
	1.54			
Ratio	1.00	0.80	0.81	0.57

TABLE 3

EFFECT OF A LARGE DOSE OF CHOLESTEROL ON THE LEUKOCYTES

Time of Taking Blood for Serum and Leukocytes	Leukocytes	Serum	Phagocytosis (Absolute Number of Bacteria per Cell)	Ratio
1 hour after injection	Rabbit A, Control 1	Rabbit A	3.46	1.00
		Rabbit B	2.26	0.67
		Rabbit C	3.34	0.96
24 hours after injection	Rabbit A, Control 1	Rabbit A	3.60	1.00
		Rabbit B	2.36	0.65
		Rabbit C	3.48	0.97
	Rabbit B, injected with 7 c.c. 0.5 percent suspension of cholesterol	Rabbit A	2.36	1.00
		Rabbit B	1.35	0.57
		Rabbit C	2.26	0.96

TABLE 4

COMPARISON OF THE ACTION OF SERUM AND OF THE LEUKOCYTES IN PHAGOCYTOSIS AFTER THE INJECTION OF A LARGE DOSE OF CHOLESTEROL

Time of Taking Blood	Normal Serum and Normal Leukocytes	Normal Serum and Corpuscles of Animal Injected with Cholesterol	Serum of Animal Injected with Cholesterol on Normal Leukocytes	Serum of Animal Injected with Cholesterol on Its Own Leukocytes
1 hour after injection	3.40	2.26
24 hours after injection	3.60	2.36	2.36	1.35
	3.48	2.26		
Ratio	1.00	0.65	0.67	0.38

cytes. The figure obtained with the serum and leukocytes of the animal injected with cholesterol shows an absolute reduction in phagocytosis of 65 percent after one hour, and of 43 percent seventy-two hours after the injection of a single small dose of cholesterol.

A large dose was then injected corresponding in quantity and concentration to some of those employed by Stuber, viz., 7 c.c. of a 0.5 percent suspension of cholesterol; 7 c.c. of distilled water plus acetone, the latter having been evaporated as in the preparation of the cholesterol suspension, were injected into control animal No. 2.

The relations of the index are approximately the same as in the experiments with a small dose, but the reduction is greater, which effect is attributed to the larger amount of cholesterol.

It is evident from these results that the action of cholesterol is not on the opsonin alone, if at all. If it were so and cholesterol would reduce the opsonic power of the serum simply by combining with the opsonin, as Arkin suggests, we would not have the constant effect of a reduced phagocytosis with normal serum on the washed leukocytes of cholesterol Rabbit B. This would rather indicate that the leukocytes have been altered by cholesterol so as to lower their phagocytic powers. Serum containing cholesterol produced the same degree of reduction in the phagocytic power of normal leukocytes within the short period of incubation as normal serum with leukocytes altered by cholesterol. This reduction is 20 to 30 percent after the small dose and 35 percent after a larger dose. The opsonic index of the serum containing cholesterol with leukocytes injured by, or acted on, by this substance is enormously diminished; the absolute reduction is 65 percent one hour after the injection of a small dose; 43 percent seventy-two hours after the same injection; and 62 percent twenty-four hours after the larger dose. These figures closely approach those obtained by Stuber. That opsonin and serum apparently have little to do with the diminished phagocytic function of the leukocytes was demonstrated by Stuber's experiments, in which the quantity of serum was reduced to a minimum. The limited number of our experiments does not permit any conclusion to be drawn as to the method in which this occurs. Besides the direct detrimental effect on the leukocytes there may be interaction with the opsonin whereby the leukocytes are injured, and there may be some effect on the bacteria; but it is not unlikely that the effect is directly and entirely on the leukocytes, as Stuber believes. This behavior of the leukocytes toward phagocytosis under the influence of cholesterol runs perhaps parallel with antihemolytic action.

Mayerstein and Allenbach¹¹ state that leukocytes are generally capable of hindering hemolysis. This action, they claim, depends on their absorption of hemolytic agents, whereby they are deviated from the erythrocytes. The absorption takes place not only by intact leukocytes, but also by broken-up cells and is perhaps chiefly dependent on their content of lipid substances. "The paralyzing effect of cholesterol on the vital functions of the leukocytes," Stuber writes, "recalls the action of the iodine ion." He showed that by substitution of the hydroxyl group of the cholesterol molecule, such as occurs in cholesterylbenzoate, cholesterol loses its inhibiting power, and the neutralizing effect of lecithin on the action of cholesterol is, according to him, due to the anchoring of the hydroxyl group of lecithin. This important group of the cholesterol molecule is therefore a chief factor in its action on the vital functions of the leukocytes. The recovery of the cells from the injury takes place after a relatively long period of time, as Stuber found phagocytosis still reduced by 50 percent on the sixth day.

Walbum obtained results which differed from those of Stuber and us; and his conclusions naturally are different. He found phagocytosis greatly increased by cholesterol, and according to him, this action is apparently not dependent on the free hydroxyl group, but rather a specific one and not an ordinary colloid phenomenon. It is not altogether impossible and his results are, in part, due to the concentration of the suspension. Dilutions with distilled water twenty times greater than those employed by Stuber and ourselves were used for the injections as well as the experiments *in vitro*. No serum nor salt solution was added to these high dilutions with distilled water. It would seem from a test *in vitro* made by Arkin that cholesterol alone has a stimulating effect on phagocytosis; without serum and without salt solution the phagocytic index of the leukocytes with cholesterol was 1.54, i. e., more than half of that of serum alone, which was 2.74. That of cholesterol with salt solution was 1.94; with serum 1.18.

In order to determine the effect of cholesterol on immunization, two weeks after the last injection our animals were used for another experiment. A blood test, made at this time, showed complete absence of cholesterol action both on leukocytes and on the serum. One cubic centimeter of a suspension of killed streptococci, containing 100,000,000 organisms, was injected into Rabbit A, and 7 c.c. of a 2 percent suspension of cholesterol were injected into Rabbit B. The same strain of streptococci, a rather virulent one, was used for phagocytosis. The serum of the rabbits was tested before and one hour after the injection; twenty-four hours following this a test was made again

11. *Biochem. Ztschr.*, 1913, 58, p. 93.

and the same injections in reversed order were made; 7 c.c. of a 2 percent suspension of cholesterol were injected into Rabbit A, which had received the vaccine, and 100,000,000 killed streptococci into Rabbit B, which had received cholesterol. One hour after these injections blood was taken for examination with the leukocytes of Rabbit C.

The figures of phagocytosis in Rabbit A, injected with killed streptococci, with serum taken before and one hour and twenty-four hours after the injection, indicate a negative phase one hour, and a decided rise twenty-four hours after the vaccine injection. A large dose of a concentrated suspension of cholesterol is followed by an enormous decrease in phagocytosis one hour after the injection, which amounts to 69 percent. Rabbit B shows a fall of phagocytosis by 64 percent

TABLE 5
THE EFFECT OF CHOLESTEROL ON PHAGOCYTOSIS IN RELATION TO IMMUNIZATION BY
KILLED STREPTOCOCCI

Animal	Opsonic Index before Injection	Dose and Order of Injection	Opsonic Index 1 Hour after Injection		Opsonic Index 24 Hours after Injection		Dose and Order of Injection	Opsonic Index 1 Hour after Injection	
			Absolute	Ratio	Absolute	Ratio		Absolute	Ratio
Rabbit A	0.52	1 c.c. = 100 million killed strep.	0.48	0.86	0.88	1.16	7 c.c. 2 percent suspension cholesterol	0.28	0.36
Rabbit B	0.52	7 c.c. 2 percent suspension cholesterol	0.20	0.36	0.58	0.76	1 c.c. = 100 million killed streptococci	0.28	0.36
Rabbit C	0.54	Control	0.56	1.00	0.76	1.00	Control	0.78	1.00

one hour after the injection of cholesterol, which after twenty-four hours is only 24 percent. One hour after the inoculation of killed streptococci the ratio of phagocytosis is reduced from 0.76 to 0.36, that is, by 53 percent.

The immediate effect of immunization on the cholesterol action and of cholesterol on immunization is striking, both resulting in a marked depression of phagocytosis, and resembles the effect which was observed in the first series of experiments on guinea-pigs, when cholesterol and killed streptococci were injected simultaneously; the curve of the indices of this animal closely follows that of the animal injected with cholesterol alone. Outside of these pronounced results, this test does not furnish us with a clue to explain the reactions. Immunization

by bacteria is supposed to increase the opsonins and we actually see a definite, tho not very marked, rise in the opsonic index twenty-four hours after the inoculation. The fall in the ratio by 69 percent one hour after the injection of a large dose of a concentrated suspension of cholesterol into the animal is slightly more than the one after an initial dose of cholesterol of the same quantity and concentration in Rabbit B, an effect, seemingly, of a complete neutralization of the influence of immunization on the opsonin. More striking is the result of a dose of killed streptococci in a rabbit which twenty-four hours previous to this has received a large dose of cholesterol; the opsonic index of 0.36 one hour after the injection had risen to 0.76. The vaccine injection is followed by a fall in phagocytosis by 53 percent. If we hold that immunization acts on the opsonin and we assume that in this case there was a marked negative phase, part of the great reduction is still to be attributed to other reactions.

In these tests of the relation of cholesterol to immunization, the condition and function of the leukocytes acted on by cholesterol and the antigenic substances are not taken into account. A consideration of these will be made later. The question of the relations between cholesterol and immunization seems to be of great interest. We are naturally led to ask: how does cholesterol, which is normally present in the blood, and in most of the organs of the human body and increased in a number of pathological conditions, act in infectious processes? Further investigations may give answer to this question that may have important bearing on therapy.

SUMMARY

It appears that cholesterol in colloidal suspension, injected into guinea-pigs and rabbits, has a depressing effect on phagocytosis.

This action is probably chiefly on the leukocytes; for the opsonic index of normal serum with the washed leukocytes of an animal injected with cholesterol is lower than that of the same serum with washed normal leukocytes, and the index of the serum containing cholesterol with the washed leukocytes of the same blood shows a reduction in phagocytosis more than twice as great as that of such serum with washed normal leukocytes.

The fall of the total phagocytic index, that is, of the serum of the animal injected with cholesterol acting on its own washed leukocytes, is 65 percent after one hour, and 43 percent seventy-two hours after

one small dose, and 62 percent twenty-four hours after one large dose of cholesterol.

The chief difference in the results from injections of small and large amounts of cholesterol and of doses of higher and lower concentration seems to be, as far as the range of these experiments indicates, a more marked immediate effect from the larger dose and from that of higher concentration.

This inhibitory action of cholesterol is perhaps due to the presence of free cholesterol in the blood.

Simultaneous injections of immunizing doses of streptococci with cholesterol result in an opsonic curve which is almost identical with that of the serum of an animal injected only with cholesterol. This apparent neutralizing effect of cholesterol on the formation of antibodies is marked when cholesterol is injected twenty-four hours after the animal has received the immunizing dose.

THE FORMATION OF CHLAMYDOSPORES IN SPOROTHRIX SCHENCKII *

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The statement has been made repeatedly by French workers, especially by Beurmann and Gougerot, that *Sporothrix schenckii* does not produce chlamydospores, whereas *Sporothrix beurmanni* does. This has been indeed one of their most important points of differentiation in their attempt to establish the existence of two distinct pathogenic sporothrices.

In his article on "Sporotrichosis" Gougerot,¹ referring to the sporothrix beurmanni, writes:

"Chlamydosporen von verschiedener Grösse und Gestalt. Sie können 4- 16- 18 μ lang werden, sind rundlich oder gestreckt, in der Länge des Fadens verteilt oder an dessen Ende, isoliert oder in Gruppen oder in Ketten von 2, 3 oder mehr Gliedern. Sie entstehen auf den Fadern selber oder auf einem kurzen besonderen Ast." Referring to the sporothrix schenckii, he writes: "Keine Chlamydosporen." Gougerot, in his paper on "North American Sporotrichosis,"² quotes Matruchot, the eminent French botanist, as follows: "Le Sporotrichium Beurmanni peut donner naissance a des chlamydospores, c'est-a-dire à des spores enkystées, munies d'une membrane épaisse. De Beurmann et Gougerot en avaient déjà signalé d'intercalaires sur le trajet des filaments mycelium et de terminales a l'extrémité de rameaux lateraux renflés; nous avons pu, nous aussi, en observer de laterales et de terminales, peut être plus caractéristiques que les intercalaires par leur forme subsphérique et par les gouttelettes huileuses qu'elles renferment. Elles apparaissent dans les vieilles cultures, dans les points où se produisent de petites touffes floconneuses de mycélium blanc; elles sont abondantes, surtout sur les portions agregées du mycélium; ce sont évidemment des organes de conservation." Referring to the sporothrix schenckii, he writes: "Pas des chlamydospores connues."

* Received for publication August 15, 1914.

1. Kolle and Wassermann Handbuch der Pathogenen Micro-organism, 2d edition, 1913, 5, p. 232.

2. Bull. et mém. Soc. méd. d. hôp. de Paris, 1910.

The strain of *Sporothrix schenckii*, referred to in these references, was sent by Hektoen to Beurmann in 1906. The observations which I wish to report in this paper were made on the same organism, a culture of which I received from Hektoen in 1910.

In the original articles by Schenck³ and by Hektoen⁴ no mention is made concerning the existence or non-existence of chlamydospores.



Fig. 1.—Chlamydospores of *Sporothrix schenckii* grown on agar 1.5 percent, NaCl 0.5 percent and water for eight weeks.

We may safely assume that they were not observed, since later studies have shown that in ordinary nutrient media they are not found in cultures of the original Hektoen strain. I wish to point out here that this strain is not unique in this respect, since, as I will show later, other strains exist which likewise do not produce chlamydospores on ordi-

3. Bull. Johns Hopkins Hospital, 1898, 9, p. 286.

4. Jour. of Exp. Med., 1900, 5, p. 77.

nary nutrient media. Certain strains, however, produce them in moderate numbers and it is this variation in strains on ordinary media that has evidently given rise to such statements as those quoted.

While observing the growth on different media of sporothrix, including, among others, strains obtained from Gougerot and Sabaraud as typical *Sporothrix beurmanni* and also the original Hektoen strain, I noted that on media poor in nutrients such, for example, as agar 1.5 percent, NaCl 0.5 percent, with or without sugar, the formation of chlamydospores was favored very decidedly. I was led, therefore, to look into this matter rather carefully, and with this point in mind a number of strains, grown on various media both rich and poor in nutrients, were examined. The strains vary considerably. Some strains, for example, a sporothrix obtained from Gougerot, one from Kren of Vienna, and one from K. F. Meyer isolated from a horse in Pennsylvania, show, on ordinary nutrient glucose agar, the presence of an occasional chlamydospore. Others, like the original Hektoen strain and a sporothrix obtained from Sabaraud in Paris, apparently do not form them in nutrient media or at least only very rarely. However, in media poor in nutrients the number of chlamydospores is very much increased. They have been observed to be numerous in the following media: Agar 1.5 + NaCl .5; agar 1.5 + asparagin 2 + $MgSO_4$ 1 + K_2HPO_4 1; either of the above with the addition of sugars, pure or impure. I have made careful notes on the eight strains which I have in my possession, and in all the formation of chlamydospores was abundant when placed on these media. There were some variations in the number of spores in the various tubes between the strains, but these were not constant; nor did they indicate any characteristic differences between the strains which could be interpreted as meaning a variety difference. I wish to suggest here that those who may have occasion to study the properties of sporothrix strains should observe whether chlamydospore formation occurs on the synthetic media mentioned.

The chlamydospores vary considerably in size, ranging from 5 microns in diameter to many times this dimension. Some appear spherical, others oblong; some are irregular and appear as tho plasmolyzed and shrunken. They are usually colorless, or only very faintly yellow. I have not seen definitely pigmented ones as the ordinary spores often appear. They occur at times in the course of a filament; again at its terminus. They are also occasionally seen attached to the side of a mycelial thread by means of a fine stalk much as are the ordinary spores. The walls are usually thick and often

irregular. They stain deeply with methylene-blue and are strongly gram-positive. Their shape is depicted in Figure 1, which is a drawing from an unstained preparation made from an eight-weeks old culture of the sporothrix schenckii. This is the strain that was obtained from Hektoen and which Gougerot has declared does not form chlamydospores.

The chlamydospores form apparently rather deep in the media where there are few or no ordinary spores, but where the mycelium is abundant. It does not appear that the oxygen supply has any material influence on their formation, for organisms grown in a limited supply of oxygen do not seem to form them more readily than when air is freely admitted. Furthermore, organisms grown on nutrient media which has been permitted to dry does not, as one might suppose, favor their formation.

It is a matter of interest that when sporothrix is planted on a medium of low nutrient value, the growth, instead of remaining largely at the surface, will penetrate deep into the culture, in time often growing well to the bottom of the tube and throughout the entire medium. It is in this mass, which can be readily seen in the media as a diffuse growth, that chlamydospore formation goes on apparently in greatest abundance. The spores may be very numerous here, at times a dozen or more being seen in a single field of the microscope.

The chlamydospores do not form in the media for some little time after distinct growth is visible. A series of tubes of the various synthetic media referred to above were inoculated and examined from time to time. After ten days a few were noted in an occasional tube; in some tubes there were none. From this time on, they gradually appeared in increasing numbers, and at the end of one month every tube contained them, some in very large numbers.

SUMMARY

The sporothrix schenckii in certain synthetic media readily forms typical chlamydospores indistinguishable from those seen in cultures of the sporothrix beurmanni.

Considerable variation in respect to chlamydospore formation exists between the various strains of sporothrices when grown on ordinary nutrient media. They all readily form them, however, when grown on synthetic media poor in nutrients.

Chlamydospore formation cannot, therefore, serve as a differentiating point between *Sporothrix schenckii* and *Sporothrix beurmanni*.

AN EXPERIMENTAL STUDY OF THE INFLUENCE OF IODIN AND IODIDS ON THE ABSORPTION OF GRANULATION TISSUE AND FAT-FREE TUBERCLE BACILLI

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. X*

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Cantacuzene,¹ in 1905, reporting an experimental study of the effects of fat-free tubercle bacilli on guinea-pigs, records that the bacilli lose much of their toxicity if treated with an iodine solution (I = 1 gm.; KI = 2 gm.; water = 400 c.c.) previous to their inoculation, and that they are then absorbed much faster than non-iodized bacilli. The resorption of fat-free bacilli and of tuberculous formations in guinea-pigs, he continues, is greatly enhanced by the daily administration of potassium iodide, the iodine salt markedly stimulating the phagocytic property of the endothelial leukocytes.

Cantacuzene's observations on the influence of iodides upon fat-free tubercle bacilli stand alone in the literature. They are especially important on account of the use of iodine and iodides in tuberculosis and in conditions where the absorption of granulation and necrotic tissue is desired. Because of this important bearing, a repetition of Cantacuzene's experiments seemed desirable, together with experiments in which agar was used to cause the formation of nodules of granulation tissue.

The human tubercle bacilli used in these experiments were obtained from Parke, Davis and Company and prepared by Dr. H. J. Corper of this laboratory. They were washed repeatedly with normal salt solution and dried. The dried bacilli were extracted in a Wiley extraction apparatus with several changes of hot absolute alcohol and several changes of warm ether, dried, pulverized in a malt mill, and again extracted with hot absolute alcohol and warm ether. The minimal lethal dose of the dried, fat-free bacilli, determined by the intraperitoneal inoculation of guinea-pigs, is about 0.04 gm. for each 100 gm. of guinea-pig weight. The agar jelly was melted and injected into the animals with a

* Received for publication August 21, 1914.

1. *Ann. de l'Inst. Pasteur*, 1905, 19, p. 699; *Compt. rend. Soc. de biol.*, 1905, 59, p. 314.

powerful paraffin syringe. All injections were made with strict aseptic precautions. The potassium iodid was given subcutaneously in 2 percent strength, this being a concentration that does not cause necrosis.

SERIES 1. CONTROLS

April 21. The guinea-pigs of this series each received 0.01 gm. of fat-free tubercle bacilli intraperitoneally.

1. Female, 450 gm., killed April 29. On the surface of the liver are two white nodules, smaller than a pin head. There are several similar nodules on the surface of the spleen and on the serous surfaces of the abdominal wall. There are many white nodules, pin head and less in diameter, in the omentum and mesentery, on the stomach, and on the inferior surface of the diaphragm.

The nodules on the liver contain bacterial masses varying in size from those just discernible with the low power to others occupying the entire center of the field. These masses are irregular in outline and surrounded by large numbers of polynuclear leukocytes outside of which is a concentric layer of epithelioid and connective tissue cells. Along the periphery there are small capillaries. A few giant cells are present in the granulation tissue.

2. Male, 470 gm., killed May 7. There are six or more white nodules, less than a pin head in size, on the upper and lower surfaces of the liver and on the spleen. In the mesentery and particularly in the omentum just below the stomach are nodules varying in size up to that of a small pea. These nodules are firm and have a rather irregularly serrated white margin. On the inferior surface of the diaphragm there are white nodules about a millimeter in diameter and on the anterior abdominal wall are somewhat larger nodules which are firmly embedded.

Sections from the nodules on the spleen, in the omentum, liver, and parietal wall have the following characteristics: They contain small, granular masses of the bacteria, irregular in size and outline; numerous polynucleated leukocytes lie around these masses, some of them with fragmented nuclei; about this aggregation of polynuclears are many large endothelial cells which, toward the periphery, have a concentric arrangement and in turn are surrounded by vascularized granulation tissue. There are small giant cells in the granulation tissue.

3. Killed May 12, 450 gm. There are small, pearly white nodules on the inferior surface of the diaphragm, on the peritoneum, in the omentum, and mesentery. There are larger nodules, 2 to 4 mm. in diameter, on the anterior abdominal wall. The spleen is mottled by nodules, 2 to 3 mm. and less in diameter.

The nodules in the omentum and mesentery consist largely of cellular elements. There are small circumscribed areas the centers of which contain granular masses of bacteria which in turn are surrounded by a large number of polynuclears and endothelial cells or epithelioid cells which toward the periphery have a concentric arrangement. The margins of the nodules consist of typical granulation tissue in which there are small capillaries. There are no well-formed giant cells in the sections studied.

4. Female, 400 gm., found dead May 20. On the parietal peritoneum are small white plaques of scar tissue. There are three or four white nodular scars on the spleen and a number on the ventral abdominal wall and inferior surface of the diaphragm. There is a single firm nodule, 3 to 4 mm. in diameter, in the omentum.

The nodules on the spleen consist chiefly of granulation tissue. In the center of rather sharply circumscribed round areas are irregular masses of bacteria surrounded by numerous polynuclears. Epithelioid and endothelial cells lie around this central portion, and, along the periphery of the areas, they assume a concentric arrangement. Vascularized granulation tissue marks the boundary of the areas, and constitutes the bulk of the intervening tissue. The nodules in the mesentery have much the same histological structure.

Summary.—Unabsorbed bacterial masses and granulation tissue are present, 39 days after the inoculation of tubercle bacilli, in guinea-pigs receiving no iodids.

SERIES 2. THE EFFECT OF IODIZED FAT-FREE TUBERCLE BACILLI INJECTED
INTRAPERITONEALLY

April 21. Each guinea-pig received 0.01 gm. of fat-free tubercle bacilli intraperitoneally. Previous to injection, the bacilli were treated for 15 minutes with 10 c.c. of an iodine solution (I=1 gm.; KI=2 gm.; water=400 c.c.). The bacilli were sedimented by centrifuging and then suspended in a sterile normal salt solution. Immediately following the inoculation the pigs gave evidence of marked abdominal irritation.

1. Male, 440 gm., found dead April 22. In the peritoneum is a large amount of clear, straw-colored fluid. On the surfaces of the liver are yellowish deposits of bacteria varying in diameter from 2 to 3 mm. to a pin point. There are similar nodules on the spleen, stomach, and scattered throughout the entire mesentery and omentum.

2. Male, 440 gm., killed May 7. There are only a few scars suggestive of nodules on the inferior surface of the diaphragm. There are dense fibrous adhesions between the liver and the stomach, and in the omentum. The parietal peritoneum is rough and irregular from fibrous scars. There are pearly white areas, several millimeters and smaller in size, from which irregular white bands of scar tissue radiate. There are a few distinct nodules in the mesentery, but they are rather soft.

The nodules on the liver are encapsulated by fibrous tissue; scattered through their interior are irregular masses of bacteria all surrounded by numerous polynuclear leukocytes. Just outside the leukocytes are many epithelial and connective-tissue cells and along the periphery are numerous budding capillaries. There are many small giant cells, which apparently have been formed by the fusion of endothelial cells, about the masses of bacteria.

3. Male, 450 gm., killed May 12. The peritoneal surfaces are roughened by fibrous tissue. There are dense fibrous adhesions between the intestinal loops as well as omental adhesions with the stomach, abdominal wall, and liver. There are only two to four distinct nodules on the peritoneum which have a diameter of from 5 to 8 mm. The surface of the spleen is mottled by white spots which are rather tough and fibrous from connective tissues. There is no free fluid in the peritoneal cavity.

The nodules are surrounded by a well-developed wall of connective tissue. In the interior of the nodules are numerous irregular granular masses of bacteria surrounded by many polynuclears. The leukocytes form the bulk of the cellular elements. There are also endothelial and small giant cells. In some places, small bacterial masses are engulfed by giant cells which apparently have

formed by the fusion of endothelial cells. Concentrically arranged epithelioid and connective-tissue cells border the polynuclear leukocytes, while throughout the nodules run septa of granulation tissue. The abundance of cellular elements and giant cells is very pronounced.

4. Male, 430 gm., killed June 12. There are dense fibrous adhesions between the anterior abdominal wall and the intestines, between the liver and diaphragm, and between the loops of the bowel. There are a few pin-head sized nodules in the mesentery. The omentum is bound down to the liver in such a way as to form a dense mass.

Microscopically, there is an abundance of cellular tissue in the nodules, especially giant cells. Many of the giant cells contain small masses of bacteria. Other larger masses of bacteria are surrounded by polynuclears. Fibrous granulation tissue is very abundant.

Summary.—There are unabsorbed bacterial masses and granulation tissue present even after 52 days. The treatment of the bacteria with iodine solution had no apparent effect on the absorption.

SERIES 3. THE EFFECT OF DAILY INJECTIONS OF POTASSIUM IODID ON THE ABSORPTION OF FAT-FREE TUBERCLE BACILLI AND GRANULATION TISSUE

Guinea-pigs 1 to 4 of this series received 0.04 gm. of potassium iodid daily and Guinea-pigs 5 to 8, 0.1 gm., all being injected intraperitoneally at the same time, April 21, with 0.01 gm. of fat-free tubercle bacilli.

1. Male, 480 gm., killed April 29. White nodules, pin head and smaller, are scattered through the omentum and mesentery. They are also present on the inferior surface of the diaphragm, on the peritoneal surfaces, the spleen, stomach, and liver.

Microscopically, in the nodules are numerous irregular masses of bacteria surrounded by a large number of polynuclear leukocytes, which form a relatively wide zone. Bordering this zone is a narrow margin of epithelioid cells and a small amount of vascular connective tissue. There are no definite giant cells.

2. Male, 460 gm., killed May 7. There are millimeter to pin point sized white nodules on the liver, stomach, and inferior surface of the diaphragm. The omentum is densely packed with firm, yellowish, or grayish-white, nodules varying in size from 3 to 4 mm. to a pin point. The spleen is normal except from 8 to 10 white nodules, 0.5 mm. and less in diameter, on its surface. There are a few nodules on the anterior abdominal wall, and here there is a firm adhesion with the left seminal vesicle. Otherwise the peritoneal surfaces are smooth. In the mesentery there are a few small nodules of variable size.

The nodules contain many small bacterial masses which have irregular outlines and are surrounded by a large number of polynuclear leukocytes at the border of which are concentrically arranged epithelioid and connective-tissue cells. The nodules are fairly well encapsulated by loose vascular connective tissue. Throughout the nodules are trabeculae of young connective tissue. Giant cells are not numerous, there being only an occasional one present which is apparently formed by the fusion of endothelial cells.

3. Female, 420 gm., killed May 12. On the inferior surface of the diaphragm and on the liver are a few small, hard, white nodules. The omentum is adherent to the abdominal wall in the region of the spleen which is also

adherent to the diaphragm. The surface of the spleen is mottled with white areas and there are a few adhesions between the intestinal loops. There is a little scar tissue on the anterior abdominal wall.

The nodules, examined microscopically contain masses of bacteria irregular in size and shape, which are immediately surrounded by a dense layer of polynuclear leukocytes. Bordering these are concentrically arranged epithelioid cells. Small capillaries are present in the tissue. The periphery of the nodule is formed by a connective-tissue capsule with numerous capillary buds. There are also occasional giant cells with three to eight nuclei, which apparently have been formed by the fusion of endothelial cells about a small mass of bacteria, or in the close vicinity of one of the larger masses.

4. Male, 410 gm., killed May 27. There are small white scars on the inferior surface of the diaphragm. The surface of the liver is spotted with yellowish-white nodules up to 2 mm. in diameter. In the omentum are nodules, 2 to 3 mm. in diameter, and smaller pearly-white ones are in the mesentery. On the ventral wall of the abdomen are nodules surrounded by scar tissue. There are adhesions between the intestinal loops and also between the loops and the anterior abdominal wall.

Masses of bacteria, irregular in size and shape, are in the nodules removed from the viscera. These masses are surrounded by polynuclear leukocytes, bordered in turn by epithelioid and young connective-tissue cells in concentric arrangement. The margin of the nodules is formed by dense connective tissue in which are well-formed capillaries and small vessels. There is much connective tissue in the nodules and frequently smaller bacterial masses are surrounded by a narrow ring of protoplasm containing numerous elongated, vesicular nuclei at either pole. Typical giant cells are numerous in the nodules.

5. Male 400 gm., killed April 29. There are many nodules, 2 mm. and smaller in diameter, scattered on the omentum, on the peritoneal surfaces, in the inferior surfaces of the diaphragm, on the liver, and the spleen.

Large and small masses of bacteria, irregular in outline and surrounded by many polynucleated leukocytes, are present in the stained sections of the nodules in the viscera. There is a thin capsule of young connective tissue about the nodules from which trabecula extend inward and appear as interrupted septa within the nodules. There are numerous endothelial cells irregularly scattered among the polynucleated leukocytes. Giant cells are not present to any great extent.

6. Male, 450 gm., killed May 8. On the inferior surface of the diaphragm, on the stomach, spleen, and liver are glistening white nodules 0.5 to 1 mm. in diameter and smaller. In the omentum are many, some 4 to 5 mm. in diameter. The omentum is densely matted together and, at the umbilicus, is adherent to the anterior abdominal wall, where there is a firm fibrous nodule the size of a small pea. Strands of connective tissue radiate about this nodule and in its center is soft caseous material. There are also other small nodules on the anterior abdominal wall and in the mesentery.

The bacillary masses in the nodules are surrounded by a large number of polynuclear leukocytes. The development of the connective tissue is fairly well advanced. It forms septa along the periphery of the cells, surrounding the masses of bacteria, as well as concentric fibers about them. About the nodules is a well-developed, but not dense, fibrous capsule.

7. Male, 430 gm., killed May 12. There are numerous nodules on the viscera and on the peritoneal surfaces varying from the very minute to almost pin

point in size. There are some fibrous adhesions between the intestinal loops. The surface of the spleen is free. All of the nodules are white and fibrous.

In the microscopic sections of the nodules are irregular masses of bacteria surrounded by polynuclear leukocytes, about which, in turn, are concentrically arranged epithelioid cells and young connective tissue. There are a few small giant cells in the sections. There is a well-defined fibrous capsule from which septa extend into the interior of the nodules. For the most part the nodule consists of polynuclear leukocytes with small proportion of endothelial cells.

8. Male, 520 gm., killed May 27. There are a few white scars on the inferior surface of the diaphragm. On the liver are several white, pin point nodules, and on the omentum there are a number, 1 to 2 mm. in diameter. There are no nodules on the anterior abdominal wall, mesentery, and intestines. Loops of the intestine are bound together by adhesions in a number of places.

The nodules in the omentum contain a great number of endothelial cells and polynuclear leukocytes, while, scattered at irregular intervals, are small granular masses of bacteria. About the larger bacillary masses are numerous polynuclear leukocytes, bordering which is a zone of endothelial cells. Typical giant cells are irregularly scattered through the nodules. There are also small trabeculae of newly-formed connective tissue.

Summary.—Unabsorbed masses of fat-free bacteria and granulation tissue are present even after 39 days with daily administration of potassium iodid in doses of 0.04 (Animals 1 to 4) and 0.1 gm. (Animals 4 to 8). The nodules are no further in organization than are those of the control animals.

SERIES 4 AND 5. THE INFLUENCE OF POTASSIUM IODID ON THE ABSORPTION OF THE FAT-FREE TUBERCLE BACILLI

April 21. Each guinea-pig in Series 4 and 5 received 0.10 gm. of fat-free tubercle bacilli intraperitoneally and Animals 1 to 4, 0.10 gm. of potassium iodid daily, Animals 5 to 8 serving as controls.

1. Male, 350 gm., found dead May 5. The body is badly decomposed. There are numerous small grayish-white nodules in the mesentery and omentum, and one is present on the liver. No microscopic study.

2. Female, 360 gm., killed May 20. There are a few white scars and several white nodules, about 1 to 2 mm. in diameter, on the inferior surface of the diaphragm. There are about six yellowish-white nodules, 2 to 3 mm. in diameter on the liver, and smaller ones are present on the serous surfaces of the stomach. The omentum is densely matted with hard nodules which have radiating bands of connective tissue about them. There are other white nodules, 3 mm. in diameter and smaller, in the mesentery and on the serosa of the bowel. On the spleen, there are three to four small white nodules, and dense fibrous adhesions bind loops of the bowel together and to the abdominal wall.

The nodules, which are surrounded by a rather thin fibrous capsule, contain bacillary masses about which polynuclear leukocytes are very abundant. These masses are irregular in size and shape. Bordering the zone of leukocytes are epithelioid cells and new connective tissue. There are a few typical giant cells in the granulation tissue, but on the whole the polynuclear leukocytes predominate.

3. Female, 620 gm., killed June 12. On the inferior surface of the diaphragm are two to four small yellowish-white nodules. There are also white areas on the liver, 2 to 3 mm. in diameter. In the omentum are many white nodules, varying in size from a pin head to 3 to 4 mm. in diameter. Fibrous bands bind a few of the intestinal loops together and to the anterior abdominal wall.

Microscopically, the small bacterial masses in the nodules are surrounded by a narrow zone of polynuclear leukocytes, while the larger masses are bordered by a rather wide zone. There are numerous giant cells in the tissue, some of which consist of a narrow margin of protoplasm forming a ring about an engulfed mass of bacteria and with nuclei lying either along the narrow periphery or collected at one end or both poles of the cell. Other giant cells are typical in appearance and contain numerous vesicular nuclei. There are many epithelioid cells and much well-developed connective tissue in the nodules. Septa of connective tissue extend through the nodules, the abundance of vascular fibrous tissue being rather striking.

4. Male, 420 gm., killed June 12. There are two or three small white nodules on the inferior surface of the diaphragm. The surfaces of the liver are free. There are large nodules, 2 to 3 mm. in diameter, in the omentum which bind the intestine together and to the anterior abdominal wall. On the surface of the spleen there are a few white areas about a millimeter and less in diameter.

There are large and small masses of bacteria in the nodules in the peritoneal cavity, the larger areas being surrounded by many polynuclear leukocytes, the smaller in part also, but usually engulfed by giant cells or in the process of being surrounded by a rim of cytoplasm. Surrounding the zone of polynuclears there are many endothelial and young connective-tissue cells which, along the border of the cell masses, have a concentric arrangement. There are many large and small giant cells as well as much vascular connective tissue in these nodules.

5. Male, 340 gm., killed May 20. There are small white scars on the inferior surface of the diaphragm, but no definite nodules. There are adhesions between the left lobe of the liver and the omentum. On the liver are only a few small nodules. The omentum is adherent to the abdominal wall in the region of the spleen and is very dense and nodular. The nodules are yellowish, opaque, and vary in size from a small pea down to a pin head and smaller. There are also adhesions about the spleen and between the intestinal loops.

In the sections prepared from the nodules in the viscera are very many giant cells. There are large irregular masses of bacteria surrounded by numerous polynuclear leukocytes, but around the smaller bacterial masses are rings of protoplasm, which are in all stages of giant-cell formation. There are also many epithelioid and young connective-tissue cells in the nodules. The fibrous capsule about the nodules is firm and dense.

6. Female, 340 gm., killed June 12. There are a few small white scars on the inferior surface of the diaphragm and also a few very small, pale areas on the surfaces of the liver and spleen. These are rather indistinct, and are not sharply differentiated from the surrounding tissues. In the omentum are numerous small white, or yellowish-white, nodules which are adherent to loops of the bowel. There are adhesions between the intestines and the anterior abdominal wall, as well as between the intestinal loops themselves. There are a few white scars on the anterior abdominal wall.

In the microscopic sections of the nodules are masses of bacteria surrounded by a large number of polynuclear leukocytes. For the most part, however, the

small masses of bacteria are engulfed by giant cells, which are abundant, frequently typically formed, but in other places having reached only partial development. Fibrous tissue and epithelioid cells are abundant.

7. Male, 410 gm., killed June 12. There are white scars on the diaphragm which mark the places where the liver is adherent. White scars, three or four in number and about 2 mm. and smaller in diameter, are scattered over the liver. There are dense fibrous adhesions between the liver, intestines, and omentum as well as intestinal adhesions with the abdominal wall. On the spleen and in the mesentery are a few white scars.

In the nodules are many giant cells which have engulfed masses of bacteria, or are in the process of so doing. There are also larger irregular masses of bacteria surrounded by polynuclear leukocytes, which in turn are bordered by fibrous tissue and epithelioid cells. The nodules have a well-developed fibrous capsule. The abundance of giant cells, epithelioid and fibrous connective-tissue cells are prominent features of these nodules.

8. Female, 400 gm., killed June 12. There are a few fibrous adhesions between the liver and the inferior surface of the diaphragm. There are large, scar-like masses in the omentum which are bound to the intestines, forming adhesions between them and the anterior abdominal wall, as well as between the intestinal loops themselves. The spleen is free and of normal appearance.

There are irregular masses of bacteria in the nodules, some of which are surrounded by polynuclear leukocytes, while others are being engulfed by giant cells. Some of the giant cells are typically formed, while others appear as rings of protoplasm about the bacterial masses. Some of the giant cells are large and have twenty to twenty-five clear, oval nuclei irregularly scattered in their cytoplasm, as well as nuclear fragments of ingested polynuclear leukocytes. There is an abundance of fibrous tissue and epithelioid cells in addition to the giant cells.

Summary.—In the controls (Animals 4 to 8) the process of absorption and organization has progressed well, but is not complete after 52 days; absorption and organization are no further advanced in Animals 1 to 4, which received potassium iodid daily.

SERIES 6. THE EFFECT OF POTASSIUM IODID ON LESIONS PRODUCED INTRAMUSCULARLY BY FAT-FREE TUBERCLE BACILLI

Nov. 25, 0.05 gm. of defatted tubercle bacilli, suspended in sterile salt solution, was injected into the large muscles of the left hind leg of two female rabbits of the same weight, one of which received from 0.08 to 0.30 gm. of potassium iodid almost daily from Nov. 25 to Feb. 24.

On Dec. 3, 0.05 mg. of defatted tubercle bacilli was injected into the large muscles of the right hind leg.

On Jan. 1, 0.05 gm. of defatted tubercle bacilli was injected into the large muscles of each front leg.

Several weeks after the injection a large nodule had developed at the site of each inoculation.

Rabbits killed and the nodules removed on Feb. 24.

The nodules removed have the following dimensions:

CONTROL RABBIT

Left hind leg—absent—a little granulation tissue.

Right hind leg—3.5x3.3x1.3 cm.

Left front leg—3.5x3.0x2.0 cm.

Right front leg—3.5x2.7x1.7 cm.

IODID RABBIT

Left hind leg—2.4x1.5x1.2 cm.

Right hind leg—2.5x2.3x1.8 cm.

Left front leg—3.5x3.0x2.5 cm.

Right front leg—3.0x2.5x3.8 cm.

Each nodule has a large caseous center and is well surrounded by a fibrous capsule.

On Dec. 3, 0.05 gm. of defatted tubercle bacilli, suspended in sterile salt solution, was injected into the large muscles of the right hind leg of two rabbits, one of which received 0.08 to 0.30 gm. of potassium iodid almost daily from Dec. 3 to March 12.

Jan. 24, injection repeated, this time into the large muscle of each front leg.

Several weeks after the injection of the bacilli a large nodule developed at the site of each inoculation.

The rabbits were killed and lesions removed on March 18.

The nodules removed have the following dimensions:

CONTROL RABBIT

Right hind leg—1.2x2.0x2.2 cm.

Right front leg—3.5x2.0x2.0 cm.

Left front leg—about the size of a small pea, probably because of faulty injection or discharge upon the surface.

IODID RABBIT

Right hind leg—1.5x2.0x3.0 cm.

Right front leg—4.0x2.5x2.5 cm.

Left front leg—4.0x3.0x1.5 cm.

All of these nodules have a caseous center and are surrounded by a firm fibrous capsule.

There is practically no difference in the rate of absorption in these animals, even tho the iodid rabbits received iodid over a considerable period of time.

SERIES 7. THE EFFECT OF IODIZED FAT-FREE TUBERCLE BACILLI WHEN INJECTED
INTRAMUSCULARLY IN GUINEA-PIGS

On Jan. 6, injected 0.05 gm. of defatted tubercle bacilli into the muscles of the hind legs of two guinea-pigs of same weight. The bacilli injected into the right leg were treated for fifteen minutes with an iodine solution ($I=1$ gm.; $KI=2$ gm.; water = 400 c.c.), sedimented by centrifuging, and suspended in sterile salt solution. The bacilli injected into the left leg were untreated.

The animals were killed on Feb. 11 and the nodules in the leg muscles removed. The dimensions of the nodules were as follows:

Guinea-pig 1. Right—2.0x1.5x1.8 cm.; left—2.0x1.3x1.0 cm.

Guinea-pig 2. Right—2.5x2.5x2.0 cm.; left—2.5x2.5x1.8 cm.

Summary.—No difference is noted in the size of the nodules produced by either the iodized or the non-iodized tubercle bacilli.

SERIES 8. THE INFLUENCE OF IODIN, IODOFORM AND IODID ON THE ABSORPTION OF AGAR

Controls.—On May 7, 1.5 c.c. of 2 percent sterile agar jelly was injected intraperitoneally in two guinea-pigs.

1. Found dead May 12. On the surface of the spleen are white areas 3 to 5 mm. in diameter. There are only suggestions of nodules in the omentum.

The nodules on the spleen consist of irregular masses of agar broken up by strands of fibrin in the meshes of which there are numerous polynuclear leukocytes and endothelial cells. Along the margin of the nodules are numerous endothelial cells, some of which have fragmented nuclei and vacuolated cytoplasm. In only a few places are giant cells beginning to form.

2. Killed May 19. There are two to four nodules of agar, 2 mm. and smaller in diameter, in the omentum. On the surface of the spleen are two or three nodules, about 1 mm. in diameter. There are a few fibrous adhesions between loops of the bowel.

In the lymphoid tissue removed from the mesentery are a few nodules which simulate a tubercle in their microscopic appearance, differing, however, in that their centers consist of a large mass of polynucleated leukocytes which form an area approximately one-fourth the diameter of the nodules. Surrounding this are concentrically arranged fibroblastic and endothelial cells. There are budding capillaries in the fibroblastic tissue as well as a few clearly defined giant cells. These small areas probably represent almost completely absorbed and organized masses of agar. On the surface of the lymph gland are small masses of agar broken up into particles by fibrin, endothelial cells, and young connective-tissue cells.

3. Killed May 28. There is a firm white nodule 5 mm. in diameter in the region of the spleen where the bowel is also adherent. There are a few pearly nodules, 1 mm. in diameter, in the mesentery, and in the omentum there is one about 2 mm. in diameter with adhesions binding it to the viscera. There is another slightly larger nodule in the intestinal loops where there are also firm fibrous adhesions. On the surfaces of the liver and spleen are a few white nodules 1 mm. in diameter.

The nodules in the viscera contain numerous small masses of agar invaded and broken up into small bits by connective tissue and endothelial cells. There are numerous giant cells in this tissue and the process of organization is well advanced. The giant cells on the whole are small, containing, on an average, from four to eight nuclei.

Iodized Agar.—In these experiments 2 c.c. of iodine solution ($I=1$ gm.; $KI=2$ gm.; water $=400$ c.c.) were added to 7 c.c. of 2 percent agar agar and 1.5 to 2.0 c.c. of the mixture injected intraperitoneally May 7.

1. Found dead May 14. There are three or four white nodules, about 3 mm. in diameter, on the spleen. No other changes were observed.

The capsule of the spleen beneath the nodules is thickened by new connective tissue, and, immediately beneath it, the tissues are hyperemic. The nodules themselves consist of masses of agar which are being broken up into smaller bits by connective tissue and endothelial cells. The process of organization on the whole is only fairly well advanced.

2. Killed May 19. The mesenteric lymph glands are enlarged, and in the mesentery there are two light-brown, rather firm masses approximately $8 \times 5 \times 3$ mm. in their greatest dimensions. On the surface of the liver and spleen are

a few pin-head and smaller sized pearly-gray nodules. There are two or three nodules about 1 mm. in diameter in the omentum.

The nodules on the surface of the spleen consist chiefly of agar which is broken up by fibrin strands in which there are also a few polynuclear leukocytes, endothelial, and connective-tissue cells. There is no very marked cellular reaction excepting along the periphery where the connective-tissue cells are relatively abundant.

3. Killed May 28. There are many pearly white nodules, 1 mm. and slightly larger in diameter, in the omentum and about the spleen. There are also numerous small fibrotic masses in the mesentery.

The splenic capsule is thickened in areas which contain small masses of agar rather completely infiltrated with endothelial cells and polynuclear leukocytes, as well as vascular connective tissue. On the surface of the splenic capsule are irregular masses of agar which are being invaded by connective tissue and endothelial cells, while in certain places the organization of the agar is fairly well advanced.

Iodoform Injections.—On May 7, 1.5 c.c. of 2 percent agar agar were injected intraperitoneally and 0.5 c.c. of a 10 percent iodoform-glycerol suspension was injected deep into the muscles of the right hind leg.

1. Killed May 19. There is one white nodule, about 2 mm. in diameter in the omentum, and there are a few smaller ones on the serous surface of the gall bladder. At the site of the inoculation there are a few adhesions with the bowel. The surfaces of the spleen and liver are free.

2. Killed May 19. There are firm adhesions between parietal peritoneum and a large mass in the omentum about $15 \times 7 \times 2$ mm. There are also numerous white nodules in the omentum, about 1.5 mm. in diameter. The mesenteric lymph glands are greatly enlarged. Iodoform is present in the tissues at the site of its injection.

The nodules in the omentum contain irregular masses of agar which, along their borders, are being infiltrated by cells chiefly of the endothelial type, but also a few polynuclear leukocytes. There the agar is broken up into smaller masses which are being engulfed by giant cells. The giant cells are numerous and in all stages of development from narrow rims of cytoplasm about the agar masses to others which simulate closely typical Langhans giant cells. The tissue, aside from that already mentioned, consists chiefly of newly-formed granulation tissue.

3. Killed May 27. There are a number of white nodules in the omentum, about 1 mm. in diameter, and also a few fibrous adhesions between the intestinal loops. Iodoform is present in the tissues at the site of the injection.

The masses of agar in the omentum are well along in the process of organization. There are a few giant cells in the granulation tissue.

Potassium Iodid.—On May 27, 1.5 c.c. of 2 percent agar agar were injected intraperitoneally and thereafter 0.04 to 0.1 gm. of potassium iodid daily.

1. Found dead May 12. There are a few nodules, 2 to 5 mm. in diameter, on the anterior abdominal wall and also a few miliary sized nodules on the omentum and on the surfaces of the spleen. There are about three to six small, thin flakes of agar free in the peritoneal cavity.

The nodules present on the viscera contain agar which is in the early stage of organization, being invaded partly by endothelial, connective-tissue cells and a few polynuclear leukocytes.

2. Found dead May 19. In the omentum there are three to six white nodules, about 1 mm. in diameter. The liver surfaces are covered by many white areas pin-head and under in size. There are also a few on the spleen. At one place, there are dense fibrous adhesions between several loops of the bowel. There is a thin flake of agar, 1 mm. in diameter, floating freely in the peritoneal cavity.

3. Killed May 27. There are a few white nodules and scars, a millimeter or so in diameter, in the omentum and on the surface of the liver. There are a number of fibrous adhesions between the loops of the bowel.

The small nodules on the liver surface are fairly well organized and contain small masses of agar infiltrated by connective tissue and endothelial cells in which are a few small giant cells with only four to six nuclei.

In the following experiments, 1.5 to 2.0 c.c. of glycerol agar (agar agar 1.5 percent; glycerol 1 percent) were injected intraperitoneally on Feb. 26 and also into the large muscle of each hind leg. Beginning Feb. 26 and thereafter 0.10 gm. of potassium iodid was given daily to Pigs 1 and 2.

1. Found dead March 13. There are only three or four thin flakes of agar agar, 2 by 3.5 mm., floating in the peritoneal cavity. There are no nodules in the mesentery, omentum, or leg muscles.

2. Found dead March 10. There are several small masses of agar 1 to 2 mm. in diameter on the surface of the liver. On the spleen there are about six or eight similar nodules 1 to 3 mm. in diameter. A small flake of agar $2 \times 2 \times 5$ mm. floats in the peritoneal cavity. In the leg muscles there is only a small granulation area about 2 to 3 mm. in diameter.

4. Killed March 13. There are only a few small masses of agar in the omentum and a few thin flakes, about 1 mm. in diameter, in the leg muscles.

5. Killed March 13. A mass of agar, 1 cm. in diameter, is present in the omentum opposite the left seminal vesicle. There are also numerous small nodules in the omentum and mesentery. There are a few small flakes in the leg muscle.

In the following experiments, 3.0 c.c. of 2 percent agar agar was injected intraperitoneally on March 3, and 0.04 to 0.10 gm. of potassium iodid was given daily to Pigs 3 and 4.

1. Killed April 20. In the omentum and mesentery, on the liver, and spleen are numerous nodules of agar, varying in size from a pin point to one in the mesentery $10 \times 8 \times 10$ mm.

2. Killed April 20. There is a large number of pin-head and smaller sized agar nodules in the mesentery, omentum, on the spleen and liver.

3. Killed April 20. In the peritoneal cavity, there is a single white mass the size of an English walnut which lies in close relation with the cecum and the small bowel. The center of this mass contains soft purulent material. There are no other nodules, the entire agar injection apparently having localized in this spot and subsequently became infected.

4. Killed April 20. There are numerous pin-head and smaller sized nodules of agar in the mesentery and omentum, on the inferior surface of the diaphragm, and on the anterior wall of the abdomen. The connective tissue reaction about these nodules seems no greater than in the controls.

Summary.—The results do not indicate that iodine, iodoform, or potassium iodid hastened the absorption of agar.

DISCUSSION

The microscopic studies of the lesions, produced in guinea-pigs by fat-free tubercle bacilli and agar jelly, disclose little difference in the intensity of the cellular reactions about the nodules whether the animals are given iodids or remain uniodized. When fat-free bacilli are used, the reaction about the bacillary masses is chiefly a polynuclear leukocytic infiltration, the abundance of the cells in a given area depending much on the size of the bacillary masses and the length of time the bacilli have been incorporated in the tissues. Endothelial and connective tissue cells gradually appear about the aggregations and, with the progressive fibrosis of the nodules, there is a gradual diminution in the leukocytes. The final destruction of the bacilli is accomplished, apparently, by endothelial phagocytes, which fuse about bacterial masses to form giant cells.

The cellular reaction about the agar jelly is, on the whole, not as active, or as marked, as it is about the masses of bacteria. There are not as many polynuclear leukocytes taking part in the organization, while the endothelial cells are relatively more abundant.

Cantacuzene reported the complete disappearance of tuberculous formations produced by 0.01 gm. of fat-free bacilli in five or six days if the guinea-pig received 0.1 gm. of potassium iodid daily, as contrasted with the lesions in control animals not receiving potassium iodid, which persisted one or two months. Among the experiments recorded here, the same conditions are repeated, but, even after forty-two days, there are well-defined, unabsorbed bacillary masses and tuberculous granulation tissue. The process of organization and absorption in the lesions has progressed no further than in the control animals. Neither do the caseous nodules produced in the leg muscles of iodized rabbits have any striking difference in size, or in degree of organization, from those in the control rabbits, even tho large doses of potassium iodid were given over a period more than three months.

The apparently increased fibrous tissue reaction about the masses of fat-free tubercle bacilli which had been treated with iodine solution, prior to their intraperitoneal inoculation, demands some explanation. Carefully analyzed, this is not difficult to understand. Heinz² injected aseptically an iodine solution into the pleural and peritoneal cavities of rabbits, and observed a fibrinous exudative inflammation in both. The masses of fat-free tubercle bacilli have a dark brown color after being removed from the iodine solution. The brown color gradually dis-

2. *Virch. Arch.*, 1899, 155, p. 44.

appears from the bacteria as the iodine diffuses into the suspending fluid if this is changed, indicating that the combination of the iodine with the bacterial protein is, at least partially physical, rather than chemical. If bacterial masses are injected into the peritoneal cavity before the iodine has diffused into the suspending liquid, but subsequently does so in the tissues, there will appear a fibrinous inflammation about the bacilli, which organizes readily and makes the appearance of a greater fibrous tissue reaction about the nodules. In the interior of these nodules, the masses of bacteria are in no further stage of absorption than are the masses in the peritoneal cavities of guinea-pigs receiving no iodine. Such guinea-pigs, as received iodized and non-iodized bacilli in opposite leg muscles, developed nodules which progressed no differently either in the extent of their development or in the rate of their absorption.

While the experimental evidence in connection with the fat-free tubercle bacilli is perhaps more extensive, that with the agar jelly is no less convincing. Here too, there is practically no difference in the degree of agar absorption and organization whether the guinea-pigs are iodized or non-iodized.

These observations have an important bearing on the prevalent idea that iodids facilitate the absorption of granulation tissue and necrotic areas. The value of iodids in tertiary syphilis cannot be denied, but here also their therapeutic action is not understood. By means of experiments, the effects of iodine and its compounds may be defined more closely and, with this knowledge, a more precise therapeutic application in various retrogressive processes becomes possible.

CONCLUSIONS

The use of iodine and iodids, in facilitating the absorption of necrotic material and organization of tuberculous and other granulation tissues, has no experimental proof.

The daily administration of potassium iodid does not hasten the removal of foreign substances, like tubercle bacilli, by stimulating the phagocytic properties of the endothelial cells. The presence of free iodine in such areas may influence the process only by favoring an inflammatory reaction, if the amount of iodine is sufficient, but has no effect in promoting absorption.

Iodized fat-free tubercle bacilli are absorbed no faster than are the non-iodized.

THE ANTIGENIC PROPERTIES OF GLYCOPROTEINS*

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INTRODUCTION (CHEMISTRY AND SOURCES OF GLYCOPROTEINS)

Many of the recent studies in immunological reactions have had to do with the antigenic properties of compound proteins. These, for the most part, have dealt with the nucleoproteins as antigens, which subject has been thoroughly reviewed by Wells.¹ The present study deals with the antigenic properties of certain mucin substances which were prepared and used as examples of that class of compound proteins known as glycoproteins. It has been shown by Wells² that a mucin prepared from the gastric mucosa of pigs is capable of acting as an efficient anaphylactogen in guinea-pigs, and this fact suggested a more complete investigation of the biological reactions of the glycoproteins.

The glycoproteins are those compound proteins which yield, on cleavage, proteins and carbohydrate complexes. It is interesting to note that Abderhalden³ has proposed dropping the name glycoproteins and would rather consider these bodies as simple proteins, because the carbohydrate group does not seem to bear the same relationship to the protein that the nucleic acid does in the nucleoproteins. Numerous chemical analyses of various mucinous substances have been made, and true mucins have been distinguished from mucoids on the basis of solubilities and precipitation properties. According to the investigations of Levene⁴ and others some so-called mucins (tendon mucin) contain chondroitin-sulphuric acid or a similar substance, and should be classified as chondroproteins. Hammarsten⁵ says that the mucin question has not been sufficiently studied to give any positive statements in regard to the occurrence of mucin. We know, however, that glycoproteins are found in two chief localities in the mammalian body: (1) as a product of epithelial cells; (2) in the interstices of connective tissue, especially of tendons. It is probable that the term mucin covers a number of related bodies among which differences exist with respect

* Received for publication August 24, 1914.

1. *Ztschr. f. Immunität.*, 1913, 19, p. 599.

2. *Jour. Infect. Dis.*, 1911, 9, p. 168.

3. *Lehrb. d. physiol. Chem.*, 1909, p. 191.

4. *Ztschr. f. physiol. Chem.*, 1901, 31, p. 395.

5. *Text Book of Physiological Chemistry* (Eng. Trans. by Mandel), 1911, 7th Ed., p. 165.

to either the protein or the carbohydrate radical, or with respect to both these components.

For the purpose of determining the antigenic properties of the glycoproteins, as a class of compound proteins, three mucin preparations were made. These preparations represent both the main sources of mucin (epithelium and connective tissue) and they are extracted from the tissues of two different species (ox and pig), so that it is possible to observe not only the antigenic property of each mucin, but also the reactions of each mucin against a different type of mucin from the same species as well as against the mucins from a different species. This involves, of course, a consideration of the questions of absolute specificity of the mucins, of biological specificity against the serum of the same species and of protein specificity independent of species.

DESCRIPTION OF PREPARATIONS

The mucin substances which were selected as typical forms are (1) ox tendon mucin, (2) ox submaxillary mucin, (3) swine stomach mucin. They were prepared and purified as well as possible according to methods which have been used by various investigators for the purpose of chemical analysis. There is some question, as there always must be with our present methods of preparation of proteins, whether each mucin preparation is a chemical individuality; and it is not certain that all other proteins were separated from the protein component of the mucin combination. The preparations represent products which have been regarded as pure mucins and for our present purpose we shall consider them as such. In their dry state they appear as white or yellowish gray powders. They are almost altogether insoluble in water but can be dissolved in large amounts of weak alkali. They give the color reactions of the proteins (biuret, Millon, Hopkins-Cole). When heated for a long time on the water bath with 2 percent hydrochloric acid or 5 percent sulphuric acid, the solution becomes dark brown. After this is cooled and made alkaline with solid potassium hydroxid, a test by Fehling's solution shows a reduction. The principle involved in the preparation of mucins is a precipitation by means of acid from water or dilute alkali extracts of the tissue. Acetic acid is used in most methods which are described in the literature for separating mucinous substances from different sources.⁶ Hammarsten has shown that precipitation by acetic acid cannot be used in the preparation of mucin from the submaxillary gland, as another protein substance is precipitated with the mucin, but this substance remains in solution if hydrochloric acid is used. Moreover, it seems best to use hydrochloric acid in all preparations of mucins since Posner and Gies⁷ have shown that when mucoid substances are precipitated with acid from tissue extracts, non-mucoid material is withdrawn from the fluid in combination with the mucoid. If more non-mucoid material remains in solution when hydrochloric acid is used than when acetic acid is used, it is plain that in seeking for products of relatively high purity the ordinary method of precipitating with acetic acid should not

6. Abderhalden, *Muzinsubstanzen*, in *Handbk. der Biochem. Arbeitsmeth.*, 1911; also *Mucin in Biochem. Handlex.*

7. *Amer. Jour. Physiol.*, 1904, 11, p. 404.

be used. Accordingly, all precipitations have been made with hydrochloric acid. In the preparation of ox tendon mucin the method of Chittenden and Gies⁸ was used. Ox submaxillary mucin was prepared after the method of Hammarsten.⁹ Swine stomach mucin seems to have been prepared usually from an extract of the stomach epithelium by precipitation with acetic acid,¹⁰ or by scraping off the crude mucinous secretion from a number of stomachs taken from fasting pigs, and purifying this by means of solution in dilute alkali and precipitation with acetic acid.¹¹ The swine stomach mucin used in the present study, however, was precipitated from an extract of the stomach linings by means of hydrochloric acid. Detailed descriptions of the methods used for the mucin preparations are as follows:

Ox tendon mucin.—Fresh Achilles tendons of oxen were freed as carefully as possible from all adherent tissues, cut into small pieces and ground in a meat grinder. The tissue was then washed in distilled water for twenty-four hours—one liter of water being used for every 500 grams. After the water was removed the tissue was washed for twenty-four hours in the same amount of 10 percent salt solution. The salt solution was then decanted and the tissue washed with running water until the chlorid was entirely removed. The tissue was next pressed as dry as possible and extracted for sixty-five hours with half saturated limewater (one liter for every 500 grams). A second extraction of forty-eight hours was also made. From these extracts, which were filtered through paper, the mucin was precipitated by the addition of large amounts of 0.2 percent hydrochloric acid. The precipitate was purified in the following manner: It was dissolved in as small an amount of 0.5 percent sodium carbonate as possible. This solution was filtered, made nearly neutral by the addition of a little 10 percent hydrochloric acid, and then precipitated by 0.2 percent hydrochloric acid. The process was then repeated. The precipitate was next washed thoroughly with a large amount of 0.2 percent hydrochloric acid, and lastly with water until the acid was entirely removed. It was then transferred to 50 percent alcohol, next to 95 percent alcohol, then to absolute alcohol, and finally to alcohol-ether in which the mucin was allowed to stand for some time with frequent changes. Dried in a vacuum desiccator over sulphuric acid, the preparation was easily reduced to a powder.

Ox submaxillary mucin.—Fresh ox submaxillary glands, after being cut as free as possible from fat and surrounding tissues, were cut up very finely by means of a meat grinder. The tissue was extracted with distilled water (one liter for every 500 grams) for thirty-six hours. This watery extract was filtered through a heavy filter with the aid of suction, and then treated with 10 percent hydrochloric acid until the strength of the liquid was 0.15 percent hydrochloric acid. On addition of the acid the mucin was precipitated, but it dissolved upon stirring. Immediately four volumes of distilled water were added and the mucin separated as a slimy mass, which could be picked up easily on a bent stirring rod. It was purified by dissolving in 0.15 percent hydrochloric acid, filtering rapidly and immediately reprecipitating by diluting with water. This process was repeated and then the mucin was thoroughly washed with water, transferred to alcohol, treated with alcohol-ether, dried, and reduced to a powder form in the same manner as described above.

8. Jour. Exper. Med., 1896, 1, p. 186.

9. Ztschr. f. physiol. Chem., 1888, 12, p. 163.

10. Abderhalden, Biochem. Handlex., p. 140.

11. Lopez-Suarez, Biochem. Ztschr., 1913, 56, p. 167.

TABLE 1
ANAPHYLAXIS EXPERIMENTS WITH OX TENDON MUCIN

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms	Days Interval	Fourth Injection	Symptoms
17	0.05 gm. ox tendon mucin	Severe with death in 1 hour and 45 minutes
18	0.05 gm. ox tendon mucin	Moderate	19	0.05 gm. ox tendon mucin	Doubtful
28	0.05 gm. ox submax. mucin	None visible; slight drop in temperature	19	0.05 gm. ox submax. mucin	None visible; slight drop in temperature	2	0.05 gm. ox tendon mucin	Moderate
30	0.05 gm. ox submax. mucin	Doubtful
16	0.05 gm. pig stomach mucin	None	19	0.05 gm. swine stomach mucin	None visible; slight drop in temperature	2	0.05 gm. ox tendon mucin	Severe
19	0.05 gm. pig stomach mucin	None	19	0.05 gm. swine stomach mucin	None visible; drop in temperature	2	0.05 gm. ox tendon mucin	Severe
29	1 c.c. ox serum	Severe with death in 1 hour
31	1 c.c. ox serum	Moderate	7	0.5 c.c. ox serum	Slight	2	0.05 gm. ox tendon mucin	Slight

Each guinea-pig received a sensitizing dose of 0.005 gm. of ox tendon mucin. Before the second injection was given an interval of at least 21 days had elapsed.

Swine stomach mucin.—Fresh swine stomach linings (the stripped mucosa obtained from the Pepsin Department of Armour & Co.) were finely chopped up by means of a meat grinder and then extracted with water for twenty-four hours (using one liter of water for every 500 grams). When the water extract was removed, the tissue was pressed as dry as possible and then extracted with the same amount of 10 percent salt solution for twenty-four hours. When the salt solution extract was removed, running water was allowed to pass through the tissue until all the sodium chlorid was removed and then an extract was made with half-saturated lime water for twenty-four hours (using one liter for every 500 grams). The lime water extract was then filtered through paper and, when about one-fourth a volume of 0.2 percent hydrochloric acid was added, the mucin precipitated. This product was collected and purified by redissolving it in 0.5 percent sodium carbonate, filtering, adding 10 percent hydrochloric acid to the neutral point, and then reprecipitating the mucin by adding an excess of 0.2 percent hydrochloric acid. This process was repeated twice. A centrifuge was used to throw down the precipitate. Finally, the mucin was washed free from acid with distilled water, treated with alcohol and alcohol-ether, and dried in the same manner as the other preparations.

In all cases the extraction was done at low temperatures and whenever it was necessary for a solution or a precipitate to stand for some time a little chloroform was added to guard against putrefaction.

THE ANAPHYLAXIS REACTIONS

Mucin does not possess a "primary" toxicity for animals into which it is injected. Wells¹² states that he has injected rabbits with large amounts of pure ox tendon mucin almost daily for two to four months without observing any ill effects. It has been mentioned that Wells has also demonstrated that pig stomach mucin is an efficient anaphylactogen; i. e., it will produce sensitization which, after a certain time, may be followed by intoxication of susceptible animals. The specificity of the anaphylaxis reaction and its value as a special biochemical method of study are well established facts and do not need to be dwelt upon here.

A few statements regarding the method of conducting experiments and describing results are perhaps necessary. Guinea-pigs of medium weight (averaging about 300 gm.) were used. The mucins were dissolved in 0.2 percent sodium hydroxid and the solution diluted with an equal volume of water before injection, making a 0.1 percent sodium hydroxid solution. A sensitizing dose of 0.005 gm. and an intoxicating dose of 0.05 gm. were used in all the experiments. In the case of serum sensitization and intoxication the doses used were 0.1 c.c. and 1 c.c. All injections were made into the peritoneal cavity. The usual special precautions regarding the thorough cleansing of syringes, glass utensils, and needles were adopted in order to avoid contamination of the injected proteins with foreign proteins. In recording the effects produced by the second or intoxicating dose as well as all subse-

12. Chemical Pathology, 1914, 2d Ed., p. 389.

TABLE 2
ANAPHYLAXIS EXPERIMENTS WITH OX SUBMAXILLARY MUCIN

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms	Days Interval	Fourth Injection	Symptoms
8	0.05 gm. ox sub-max. mucin	Severe with death in 37 minutes	..	0.05 gm. ox sub-max. mucin
9	0.05 gm. ox sub-max. mucin	Severe
10	0.05 gm. ox tendon mucin	None	2	Severe
11	0.05 gm. ox tendon mucin	None
13	0.05 gm. pig stomach mucin	None	19	0.05 gm. swine stomach mucin	None visible; drop in temperature
14	0.05 gm. pig stomach mucin	Doubtful	19	0.05 gm. swine stomach mucin	None
12	1 c.c. ox serum	Moderate	7	0.5 c.c. ox serum	None	2	0.05 gm. ox sub-max. mucin	Moderate
15	1 c.c. ox serum	Slight

Each guinea-pig received a sensitizing dose of 0.005 gm. of ox submaxillary mucin. Before the second injection was given an interval of at least 21 days had elapsed.

TABLE 3
ANAPHYLAXIS EXPERIMENTS WITH PIG STOMACH MUCIN

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms	Days Interval	Fourth Injection	Symptoms
21	0.05 gm. pig stomach mucin	Moderate
23	0.05 gm. pig stomach mucin	Moderate
20	0.05 gm. ox tendon mucin	None	19	0.05 gm. ox tendon mucin	None visible; slight drop in temperature	2	0.05 gm. pig stomach mucin	Severe with death in 18 hours
22	0.05 gm. ox tendon mucin	None
24	0.05 gm. ox sub-max. mucin	None	19	0.05 gm. ox sub-max. mucin	Marked drop in temperature	2	0.05 gm. pig stomach mucin	None
27	0.05 gm. ox sub-max. mucin	Slight	19	0.05 gm. ox sub-max. mucin	Severe with death in 1 hour and 4 minutes
25	1 c.c. pig serum	Severe with death in 20 minutes*
26	1 c.c. pig serum	Severe

Each guinea-pig received a sensitizing dose of 0.005 gm. of pig stomach mucin. Before the second injection was given an interval of at least 21 days had elapsed.

*This pig found to be in a late stage of pregnancy at autopsy.

quent doses, the following terms were used with the constant significance, as given by Wells:¹³

"1.—'Doubtful' symptoms. The animal scratches itself a few times and perhaps seems a little uneasy or a trifle ill; the temperature remains normal or falls not more than 1-1.5 C. Such results may be occasionally observed after injection of foreign proteins into non-sensitized animals, and are not regarded as of any significance.

"2.—'Slight' symptoms. The animal does not become seriously ill, but scratches itself vigorously, is either very restless or very somnolent or both alternately, hair roughened, eyes usually lachrymate and partly closed. The temperature usually falls 1-1.5 C. Such a reaction is believed to indicate usually, if not always, a real sensitization and a true anaphylactic intoxication, but it may possibly sometimes, but rarely, occur from foreign proteins without sensitization.

"3.—'Moderate' symptoms. More seriously ill than under 2. Usually the hair becomes very rough, with respiratory distress and marked lachrymation, fall of temperature of 1.5-3.5 C.; frequently the animal lies down and sometimes there is violent coughing.

"4.—'Severe' symptoms. Same as under 3, but so much more severe as to threaten to cause death, but followed by complete recovery in a few hours. In these animals the temperature usually falls 3 to 4 degrees or even more."

TABLE 4
ANAPHYLAXIS EXPERIMENTS WITH OX SERUM

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms
1	0.05 gm. ox tendon mucin	None
5	0.05 gm. ox tendon mucin	Doubtful	2	0.5 c.c. ox serum	Moderate
7	0.05 gm. ox tendon mucin	Slight	3	0.5 c.c. ox serum	Moderate
2	0.05 gm. ox sub-max. mucin	Doubtful	3	0.5 c.c. ox serum	Severe
3	0.05 gm. ox sub-max. mucin	Slight	2	0.5 c.c. ox serum	Severe
4	0.05 gm. ox sub-max. mucin	Slight

Each guinea-pig received a sensitizing dose of 0.1 c.c. of ox serum. Before the second injection was given an interval of at least 21 days had elapsed.

TABLE 5
ANAPHYLAXIS EXPERIMENTS WITH SWINE SERUM

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms
32	0.05 gm. swine stomach mucin	Doubtful	5	0.5 c.c. swine serum	Moderate
33	0.05 gm. swine stomach mucin	Slight	5	0.5 c.c. swine serum	Slight

Each guinea-pig received a sensitizing dose of 0.1 c.c. of pig serum. Before the second injection was given an interval of at least 21 days had elapsed.

These experiments show that with each mucin it was possible to produce anaphylactic intoxication in sensitized guinea-pigs, the reaction varying in degree from moderate symptoms to death. About the same reactions were obtained in guinea-pigs sensitized with the mucin when an intoxicating dose of blood-serum from the same species was given. The evidence is very slight that sensitization occurred when a guinea-pig was injected with one mucin preparation so that it would react positively upon receiving an intoxicating dose of the other mucin preparations from the same species as well as from a different species. However, a comparison of the records for Guinea-pigs 28 and 16 (Table 1) shows that, when subsequent injections were made for the purpose of determining the presence of a refractory period, the anti-anaphylactic condition was greater in the case of ox tendon mucin against ox submaxillary mucin than it was in the case of ox tendon mucin against pig stomach mucin. In these cases, while the symptoms following the third injection noted are "none visible," yet a drop in temperature was observed which alone might be taken to indicate a positive reaction; such a reaction can easily be accounted for by a sensitization with the second injection, as there was an interval of nineteen days between the injections. All the animals sensitized with either ox serum or pig serum did not react positively, or, at the most, only doubtfully or slightly so, to the mucins from the same species in each case, and the mucins did not render them refractory to the sera. On the basis of these experiments it might be interpreted that some traces of blood proteins were present in the mucin preparations.

THE PRECIPITIN REACTIONS

In order to study the precipitin reactions, young full-grown male rabbits were selected for the production of immune sera. The method first advocated by Fornet and Müller¹⁴ was used. These investigators and others¹⁵ have shown that when rabbits are given intraperitoneal injections of increasing amounts of foreign serum on three successive days there results a considerable production of precipitins. Hektoen¹⁶ has obtained the same results and he shows by charts that the acme of antibody content in the serum is reached about the twelfth day after the last injection. The doses of serum used are usually 5, 10, and 15 c.c.

14. *Ztschr. f. biol. Technik u. Methodik*, 1908, 1, p. 201.

15. Bonhoff and Tsuzuki, *Ztschr. f. Immunitätsf., Orig.*, 1910, 4, p. 180. Gay and Fitzgerald, *Univ. Cal. Publ. in Path.*, 1912, 2, p. 77.

16. *Jour. Infect. Dis.*, 1914, 14, p. 403.

TABLE 6
PRECIPITIN TEST WITH OX TENDON MUCIN

Dilution of Antigen 1 Percent Solution	Ox Tendon Mucin Antiserum	Normal Rabbit Serum	Ox Submax. Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum	No Antiserum
0.5 c.c.	Turbid and ppt.	Clear	Trace ppt.	Trace ppt.	Turbid	Clear	Clear	Clear
0.25 c.c.	" " "	Clear	Trace ppt.	Slight ppt.	Trace ppt.	Clear	Clear	Clear
0.125 c.c.	" " "	Clear	Clear	Trace ppt.	Slight ppt.	Clear	Clear	Clear
0.0625 c.c.	" " "	Clear	Trace ppt.	Trace ppt.	Trace ppt.	Clear	Clear	Clear
0.03125 c.c.	Slight ppt.	Clear	Trace ppt.	Trace ppt. (?)	Trace ppt.	Clear	Clear	Clear
0.0156 c.c.	" " "	Clear	Turbid	Clear	Turbid	Clear	Clear	Clear
0.0078 c.c.	Trace ppt.	Clear	Clear	Clear	Trace ppt.	Clear	Clear	Clear
0.0039 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
0.0019 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
0.0009 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
No antigen	Clear	Clear	Clear	(Clear)	(Clear)	Clear	Clear	Clear

TABLE 7
PRECIPITIN TEST WITH OX SUBMAXILLARY MUCIN

[illegible]

injected one day apart. Accordingly, the preparation of the immune sera used in the present study was carried out as follows:

Rabbits were injected intraperitoneally with ox tendon mucin, ox submaxillary mucin, pig stomach mucin, ox serum, swine serum, and crystallized egg albumin. The doses of mucin used were 0.5, 1, and 1.5 gm. which were dissolved in as small amounts of 0.1 percent sodium hydroxid as possible. The same amounts of crystallized egg albumin were dissolved in water. The sera were injected in doses of 5, 10, and 15 c.c. In all cases, the triple injections were given one day apart. On the twelfth day (in two instances it was the fourteenth) after the last injection, the animals were bled to death from the carotid under ether anesthesia, the blood was allowed to clot and the serum was pipetted off and hermetically sealed in tubes. These were kept in

TABLE 9

PRECIPITIN TEST WITH OX SERUM

Dilution of Antigen	Ox Serum Antiserum
0.05 c.c.	Marked ppt.
0.025 c.c.	Marked ppt.
0.0125 c.c.	Marked ppt.
0.00625 c.c.	Ppt.
0.003125 c.c.	Turbid
0.00156 c.c.	Ppt.
0.00078 c.c.	Slight ppt.
0.00039 c.c.	Slight ppt.
0.00019 c.c.	Slight ppt.
0.00009 c.c.	Trace ppt.
0.000045 c.c.	Trace ppt. (?)
No antigen	Clear

No precipitate with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, pig stomach mucin antiserum, pig serum antiserum, egg albumin antiserum, and no antiserum.

TABLE 10

PRECIPITIN TEST WITH PIG SERUM

Dilution of Antigen	Pig Serum Antiserum
0.05 c.c.	Turbid
0.025 c.c.	Turbid
0.0125 c.c.	Turbid
0.00625 c.c.	Ppt.
0.003125 c.c.	Ppt.
0.00156 c.c.	Slight ppt.
0.00078 c.c.	Slight ppt.
0.00039 c.c.	Slight ppt.
0.00019 c.c.	Trace ppt.
0.00009 c.c.	Clear
0.000045 c.c.	Clear
No antigen	Clear

No precipitate with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, pig stomach mucin antiserum, ox serum antiserum, egg albumin antiserum and no antiserum.

the refrigerator until they could be used in making the tests. The crystallized egg albumin was used to produce an antiserum which served as a control in that it represented the antibody of a pure protein which is known to act as a powerful antigen. Inasmuch as it was necessary to dissolve the mucin preparations in 0.1 percent sodium hydroxid before the antigenic dilutions could be made for each test, the egg albumin was also used to control the matter of whether or not the small amounts of alkali interfered with the precipitin reaction, by making antigenic mixtures for the egg albumin precipitin test with both 0.1 percent sodium hydroxid and water. Normal rabbit serum (for control) and antiserum were always used in 0.1 c.c. amounts; the several antigens in variable dilutions made up to 0.5 c.c. The antigen mixture in the case of the mucins was a 1 percent solution in 0.1 percent sodium hydroxid with a total volume of 0.5 c.c., all dilutions being made with 0.85 percent salt solution. Observations were taken after two hours' incubation at 37 C. and again after a night's stand in the ice-box. The results of the tests are given in the following tables.

While the anaphylaxis experiments demonstrated the antigenic properties of the mucins and indicated a certain specificity, the precipitin tests revealed the same facts much more clearly on account of the quantitative nature of the reactions. Each mucin produced antibodies which reacted with itself in comparatively high dilutions, but did not seem to be as powerful an antigen as the pure simple protein (crystallized egg albumin) which was tested under the same conditions as a control. Table 6 shows that the antiserum obtained from injecting ox tendon mucin into a rabbit reacts fairly well with that glycoprotein; it reacts less markedly with another glycoprotein from the same species, namely, ox submaxillary mucin; and it reacts definitely, but in a still smaller degree, with a mucin from a different species, viz., swine stomach mucin. It also reacts with the blood serum of the same species, the ox, in just as high dilutions as it reacts with either of the heterologous mucins, but it does not react at all with the blood serum of the different species, the swine. The same quantitative differences seem to hold for all three of the mucin preparations. While it was possible to interpret some of the results obtained from the anaphylaxis experiments as being due to traces of blood proteins in the mucin preparations, such an interpretation can hardly be placed upon the precipitin tests. It need scarcely be repeated here that great care was exercised in making the mucin preparations and that these products, representing glycoproteins from two sources (connective tissue and epithelium) taken from two species (ox and swine) all possessed practically the same physical properties and all gave the ordinary protein color reactions as well as yielding a carbohydrate radical on cleavage, yet in their antigenic properties they exhibited a quantitative protein specificity independent of source and species. Table 7 shows no precipitation when swine stomach mucin antiserum was added to solutions containing ox submaxillary mucin, but it is likely that in this case the test was not delicate enough to detect an antigen-antibody reaction, as the following complement-fixation tests would seem to indicate. If there is a species specificity among these glycoproteins it is very much less apparent than the protein specificity. However, in all cases, the reaction of the mucin used in immunization is produced to a greater degree with the antiserum of another mucin from the same species, than it is with the antiserum of a mucin from a different species. These results would seem to support the view that specificity depends on the chemical composition of the antigenic protein, rather than its biological origin. But as

TABLE 11
PRECIPITIN TEST WITH CRYSTALLIZED EGG ALBUMIN

Dilution of Antigen 1 Percent Solution	Egg Albumin Antiserum NaOH Solution of Antigen	Egg Albumin Antiserum H ₂ O Solution of Antigen	Normal Rabbit Serum NaOH Solution of Antigen	Ox Tendon Mucin Antiserum NaOH Solution of Antigen	Ox Submax. Mucin Antiserum H ₂ O Solution of Antigen	Pig Stomach Mucin Antiserum H ₂ O Solution of Antigen	Ox Serum Antiserum NaOH Solution of Antigen	No Antiserum H ₂ O Solution of Antigen
0.5 c.c.	Slight ppt.	Ppt.	Clear	Slightly turbid	Slightly turbid	Slightly turbid	Clear	Clear
0.25 c.c.	Slight ppt.	Ppt.	Clear	Slightly turbid	Slightly turbid	Slightly turbid	Clear	Clear
0.125 c.c.	Ppt.	Ppt.	Clear	Slightly turbid	Slightly turbid	Slightly turbid	Clear	Clear
0.0625 c.c.	Ppt.	Ppt.	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.03125 c.c.	Ppt.	Slight ppt.	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.0156 c.c.	Ppt.	Slight ppt.	Clear	Clear	Slightly turbid	Slightly turbid	Clear	Clear
0.0078 c.c.	Ppt. and turbid	Ppt. and turbid	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.0039 c.c.	Ppt. and turbid	Ppt. and turbid	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.0019 c.c.	Ppt. and turbid	Slight ppt.	Clear	Clear	Slightly turbid	Clear	Clear	Clear
0.0009 c.c.	Turbid	Slight ppt.	Clear	Clear	Slightly turbid	Clear	Clear	Clear
No antigen	Clear	Clear	Clear	Clear	Slightly turbid	Clear	Clear	Clear

Wells¹⁷ has said, "Whether the chemical differences that determine specificity are of quantitative nature, which can be disclosed by analytic means, or whether they are sometimes dependent upon spatial relationships of the amino-acid radicals, as Pick suggests, remains to be determined."

THE COMPLEMENT-FIXATION REACTIONS

The complement-fixation reaction was found a more delicate test for antibodies than the precipitin reaction, as is usually the case. The tests were made with the same amounts of antigen and immune serum that were used in the precipitin reactions. The details of the technic follow:¹⁸

TABLE 12
COMPLEMENT-FIXATION TEST WITH OX TENDON MUCIN

Dilution of Antigen 1 Percent Solution	Ox Tendon Mucin Antiserum	Normal Rabbit Serum	Ox Submax. Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	—	+	Partial +	+	—	} Complete hemolysis	
0.25 c.c.	—	+	Partial +	+	—		
0.125 c.c.	—	+	Partial +	+	—		
0.0625 c.c.	—	+	Partial +	+	—		
0.03125 c.c.	—	+	+	+	Trace +		
0.0156 c.c.	—	+	+	+	Trace +		
0.0078 c.c.	—	+	+	+	Trace +		
0.0039 c.c.	—	+	+	+	Trace +		
0.00195 c.c.	—	+	+	+	Trace +		
0.00097 c.c.	—	+	+	+	Trace +		
0.00048 c.c.	Trace +	+	+	+	Partial +		
0.00024 c.c.	Trace +	+	+	+	Partial +		
0.00012 c.c.	Trace +	+	+	+	Partial +		
0.00006 c.c.	Trace +	+	+	+	+		
0.00003 c.c.	Partial +	+	+	+	+		
No antigen	+	+	+	+	+		

Antigenic mixtures.—One percent solution in 0.1 percent sodium hydroxid with a total volume of 0.5 c.c. Serum used for antigen was heated (56 C. for thirty minutes).

Antiserum.—0.1 c.c. (56 C. for thirty minutes).

Complement.—0.05 c.c. of mixed serum from two guinea-pigs. This was never more than twenty-four hours old. Dilution to 0.1 c.c. in salt solution. Incubation with antigen and antibody at 37 C. for one hour.

Hemolytic system.—1 c.c. of a 5 percent suspension of washed sheep corpuscles containing two minimal hemolytic doses of a strong rabbit anti-sheep serum (1 c.c. of 1:1,000).

Positive Fixation.—Complete absence of hemolysis after two hours at 37 C. followed by sedimentation over night in the ice-box.

17. Chemical Pathology, 1914, 2d Ed., p. 187.

18. Gay and Robertson, Jour. Exper. Med., 1912, 16, p. 476.

Controls.—Each antigenic dilution plus 0.1 c.c. of inactivated normal rabbit serum. Antiserum with salt solution replacing antigen. These should hemolyse completely.

The results of the tests are recorded in the terms of degrees of hemolysis as follows: + = complete hemolysis; — = no hemolysis; trace + = trace of hemolysis; partial + = partial hemolysis, fifty percent or more; almost + = almost complete hemolysis.

These results show specific antigen-antibody reactions in higher dilutions than observed in the precipitin tests. On the whole, the quantitative nature of the reactions is the same. In the few instances where the results of the precipitin and complement-fixation tests do not harmonize, the lack of agreement may be accounted for by the difference in delicacy of the reactions or some slight error in manipulation. Sufficient amounts of the antisera were not on hand to repeat the tests.

TABLE 13
COMPLEMENT-FIXATION TEST WITH OX SUBMAXILLARY MUCIN

Dilution of Antigen 1 Percent Solution	Ox Submax. Mucin Antiserum	Normal Rabbit Serum	Ox Tendon Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	—	+	Trace +	Trace +	Trace +	} Complete hemolysis	
0.25 c.c.	—	+	Trace +	Trace +	Trace +		
0.125 c.c.	—	+	Trace +	Partial +	Trace +		
0.0625 c.c.	—	+	Trace +	Partial +	Trace +		
0.03125 c.c.	—	+	Partial +	Partial +	Partial +		
0.0156 c.c.	—	+	Partial +	Partial +	Partial +		
0.0078 c.c.	—	+	Partial +	Partial +	Partial +		
0.0039 c.c.	—	+	Partial +	Partial +	Almost +		
0.00195 c.c.	Trace +	+	Partial +	Partial +	Almost +		
0.00097 c.c.	Trace +	+	Partial +	Partial +	Almost +		
0.00048 c.c.	Partial +	+	Partial +	+	Almost +		
0.00024 c.c.	Partial +	+	Partial +	+	Almost +		
0.00012 c.c.	Partial +	+	Almost +	+	Almost +		
0.00006 c.c.	+	+	+	+	Almost +		
0.00003 c.c.	+	+	+	+	Almost +		
No antigen	+	+	+	+	+		

SUMMARY

A study of the results obtained from anaphylaxis experiments, as well as precipitin and complement-fixation tests, leads to the following conclusions in respect to the antigenic properties of the glycoproteins, ox tendon mucin, ox submaxillary mucin, and swine stomach mucin, which preparations were made and purified as well as possible according to standard methods.

The glycoproteins are capable of acting as antigens, but are not as powerful in their antigenic properties as simple proteins. This is in accordance with what other investigators have found to be true of

TABLE 14
COMPLEMENT-FIXATION TEST WITH SWINE STOMACH MUCIN

Dilution of Antigen 1 Percent Solution	SwineStomach Mucin Antiserum	Normal Rabbit Serum	Ox Tendon Mucin Antiserum	Ox Submax. Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	—	+	—	Trace +	+	Trace +	+
0.25 c.c.	—	+	—	Trace +	+	Trace +	+
0.125 c.c.	—	+	Trace +	Trace +	+	Trace +	+
0.0625 c.c.	—	+	Trace +	Trace +	+	Trace +	+
0.03125 c.c.	—	+	Partial +	Slight +	+	Partial +	+
0.0156 c.c.	—	+	+	+	+	Partial +	+
0.0078 c.c.	—	+	+	+	+	Almost +	+
0.0039 c.c.	—	+	+	+	+	Almost +	+
0.00195 c.c.	—	+	+	+	+	Almost +	+
0.00097 c.c.	—	+	+	+	+	Almost +	+
0.00048 c.c.	Partial +	+	+	+	+	Almost +	+
0.00024 c.c.	+	+	+	+	+	Almost +	+
0.00012 c.c.	+	+	+	+	+	Almost +	+
0.00006 c.c.	+	+	+	+	+	Almost +	+
0.00003 c.c.	+	+	+	+	+	Almost +	+
No antigen	+	+	+	+	+	+	+

TABLE 15
COMPLEMENT-FIXATION TEST
WITH OX SERUM

Dilution of Antigen	Ox Serum Antiserum
0.05 c.c.	Trace +
0.025 c.c.	—
0.0125 c.c.	—
0.00625 c.c.	—
0.003125 c.c.	—
0.00156 c.c.	—
0.00078 c.c.	—
0.00039 c.c.	—
0.000195 c.c.	—
0.000097 c.c.	—
0.000048 c.c.	Trace +
0.000024 c.c.	Trace +
0.000012 c.c.	Partial +
0.000006 c.c.	Partial +
0.000003 c.c.	Almost +
No antigen	+

Complete hemolysis with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, swine stomach mucin antiserum, swine serum antiserum, and egg albumin antiserum.

TABLE 16
COMPLEMENT-FIXATION TEST
WITH SWINE SERUM

Dilution of Antigen	Swine Serum Antiserum
0.05 c.c.	—
0.025 c.c.	—
0.0125 c.c.	—
0.00625 c.c.	—
0.003125 c.c.	—
0.00126 c.c.	—
0.00078 c.c.	—
0.00039 c.c.	—
0.000195 c.c.	—
0.000097 c.c.	—
0.000048 c.c.	—
0.000024 c.c.	—
0.000012 c.c.	Trace +
0.000006 c.c.	Partial +
0.000003 c.c.	Almost +
No antigen	+

Complete hemolysis with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, swine stomach mucin antiserum, ox serum antiserum, and egg albumin antiserum.

TABLE 17
COMPLEMENT-FIXATION TEST
WITH CRYSTALLIZED EGG
ALBUMIN

Dilution of Antigen 1 Percent Solution	Egg Albumin Antiserum
0.5 c.c.	Trace +
0.25 c.c.	—
0.125 c.c.	—
0.0625 c.c.	—
0.03125 c.c.	—
0.0156 c.c.	—
0.0078 c.c.	—
0.0039 c.c.	—
0.00195 c.c.	—
0.00097 c.c.	—
0.00048 c.c.	—
0.00024 c.c.	—
0.00012 c.c.	Partial +
0.00006 c.c.	Partial +
0.00003 c.c.	Almost +
No antigen	+

Complete hemolysis with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, pig stomach mucin antiserum, ox serum antiserum, and pig serum antiserum.

other compound proteins as well as simple proteins modified by the addition of various substances.

Each mucin gives rise to an antiserum that reacts with itself in comparatively high dilutions; with the blood serum of the same species to a less degree; and with the other mucins almost as well as with the blood serum. The reaction with the other mucins is independent of species and the antiserum does not react with the blood serum of the different species.

The present study does not throw any light on the nature of the union of the protein and carbohydrate components of the glycoproteins, excepting that it demonstrates that the antigenic properties of this class of compound proteins are different from the antigenic properties of another common class of compound proteins, the "nucleoproteins," which as such are said to produce specific antibodies for themselves. While the antibodies produced by the mucins do react with the blood serum of the same species, still there are very marked quantitative differences in favor of the homologous mucin. In so far as the antibodies produced by one mucin react with other mucins from a different species as well as from the same species, support is given to the theory that specificity depends on the chemical nature of the antigenic protein rather than its biological origin.

THE THERAPEUTIC VALUE OF COPPER AND ITS DISTRIBUTION IN THE TUBERCULOUS ORGANISM

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY OF TUBERCULOSIS. XI*

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In 1911 the study of a number of problems bearing on the chemotherapy of tuberculosis was begun. Among them was included the study of various salts of copper to determine (1) whether or not copper possessed any bactericidal action toward the tubercle bacillus, (2) whether or not it in any way influenced the progress of the disease in the animal organism or, in other words, was of therapeutic value, and (3) its distribution in the tuberculous organism.

The reasons for choosing copper and its salts were that it represents the large class of chemical substances, the heavy metals, which have proved of vast importance biologically; that it has a reputed value in the treatment of diseases due to higher fungi, such as idiomycosis, actinomycosis, and sporotrichosis, and that it possesses supposedly high fungicidal and bactericidal properties as compared to its low toxic properties.

The work was well under way and results at hand when, in 1912, there appeared three papers with rather striking results from the use of copper in tuberculosis by Dr. Gräfin von Linden,¹ E. Meissen,² and A. Strauss.³ Shortly afterwards a controversial paper appeared by Selter⁴ who, tho associated with von Linden in the experimental work, did not agree with her conclusions. A second paper by von Linden⁵ appeared a little later practically repeating the first paper. Strauss⁶ then reported on the treatment of skin tuberculosis with favorable results, in seventy cases, from the use of compounds of copper and iod-methylene blue, made according to a special process by Farbenfabrik vorm. Fr. Bayer & Company. All of these papers were considered more fully in a preliminary report as a result of some of the work included in this paper.⁷

* Received for publication August 24, 1914.

1. Beitr. z. Klin. d. Tuberk., 1912, 23, p. 201.

2. Ibid., p. 215.

3. Ibid., p. 223.

4. Ibid., 24, p. 261.

5. München. med. Wchnschr., 1912, 59, p. 2560.

6. Ibid., p. 2718.

7. Corper, DeWitt, and Wells: Jour. Am. Med. Assn., 1913, 60, p. 887.

Strauss⁸ reports further cases of external tuberculosis treated with the copper lecithin compounds and iod-methylene blue with striking improvement. Feldt⁹ states that copper, in the forms of copper chlorid and copper sulphate, inhibits growth only in a dilution of 1:5,000 on asparagin media. DeWitt found that tubercle bacilli can be suspended for days in a 1 percent solution of copper chlorid without evident decrease in their virulence for guinea-pigs.¹⁰

Bodmer¹¹ gave copper treatment to about twenty cases of pulmonary tuberculosis, mostly severe cases, and believes that, in some, benefit was obtained. Pekanovich¹² tried giving copper intravenously, at frequent intervals, in 18 cases and found it had no influence on the fever or the other symptoms of pulmonary tuberculosis. His associate, Somagyi, tested the copper in salve form for two months on cases of lupus and tuberculosis verrucosa cutis without benefit. Eggers¹³ treated fifteen cases of internal tuberculosis using the von Linden preparations supplied by Bayer and Company, and according to their directions, and states that the results in these cases were questionable. Five cases of external tuberculosis were treated with beneficial results. Pohl¹⁴ used the von Linden therapy in eighteen cases of internal tuberculosis, giving internal (capsule), intravenous, and inunction treatments, with negative results. He suggests that some other form of copper may prove valuable but that this treatment was of no value.

Von Linden¹⁵ states that she found copper salts to be equal to gold (which Feldt had found to inhibit growth in concentrations from 1:100,000 to 1:2,000,000) in their power to inhibit growth. The tubercle bacilli, according to von Linden, have a special affinity for copper salts and methylene-blue and absorb these in large amounts from watery solutions. If media containing copper are planted with tubercle bacilli, the cultures (1-2 days after inoculation) turn a green or reddish color, this accumulation even occurring in a dilution of 1:1,000,000. Organisms from the air contaminating the media remain uncolored, indicating a special affinity of the tubercle bacillus for copper, not possessed by molds and air bacteria. A like affinity for copper, as demonstrated chemically, is also shown by tuberculous tissues according to the first paper by von Linden. If the tuberculous lung of a recently killed animal is placed in a copper lecithin solution of 1:100,000 copper content for 24 hours at 37 C., the tubercles are colored green, while the healthy lung gets a copper haemol color. The green color is due to deposited copper salts. In cultures on artificial protein media, copper chlorid and copper lecithin inhibit growth in concentrations of 1:1,000,000. As 1:100,000 kills the cultures, transfer to fresh media or inoculation into animals gives negative results. Copper lecithin media are more active in this respect than media containing copper chlorid. It requires 0.4 mg. copper as copper chlorid in dilution of 1:2,000 to kill 1 mg. of tubercle bacilli in 24 hours. In five hours the bacilli were weakened to a considerable extent. Copper cinna-mate in cod-liver oil proved to be more active in this respect.

Finally, Strauss¹⁶ tried to explain various theoretical points in the use of Lecutyl (a proprietary name for the copper lecithin preparations put on the market by Farbenfabriken vorm. Fr. Bayer & Company) and described his method of treating external tuberculosis.

8. Deutsch. med. Wchnschr., 1913, 39, p. 503.

9. Ibid., p. 549.

10. Ztschr. f. Chemoth., 1913-14, 2, p. 126.

11. Munchen. med. Wchnschr., 1913, 60, p. 1756.

12. Deutsch. med. Wchnschr., 1913, 39, p. 1352.

13. Beitr. z. klin. Tuberk., 1913, 29, p. 261.

14. Wien. klin. Wchnschr., 1914, 27, p. 96.

15. Munchen. med. Wchnschr., 1914, 61, p. 586.

16. Ztschr. f. Chem., 1914, 2, p. 171.

TOXICITY OF COPPER SALTS OF AMINO ACIDS

The copper amino acid mixtures, a combination of copper with the "bausteine" of the protein molecule, which were used in many of our experiments, would seem, in many ways, to have decided advantages over the ordinary inorganic copper salts for introduction into the animal organism; there at least appeared to be no logical objection to their use. The mixtures were made by hydrolizing with sulphuric acid different proteins (egg white and human placenta) until no biuret reaction was given, removing the H_2SO_4 with $\text{Ba}(\text{OH})_2$ and any free ammonia by boiling, and then saturating with fresh copper oxid all the amino acids present. Theoretically, the mixtures consisted of the copper salts of all the amino acids present in the proteins used. Since no observations are available on the toxicity of these mixtures, it was found necessary to determine this. Comparisons were made with a copper salt, copper sulphate, that had been previously thoroughly studied. The systemic toxicity was tested in mice, and it was found that the systematic toxicity of copper sulphate and the copper amino acid mixtures based on equal copper contents is about the same, the lethal dose being between 0.05 and 0.1 mg. copper intraperitoneally for a 10 gm. mouse. When given intramuscularly, absorption is uncertain on account of the protein combinations formed by copper in the tissues, and comparable results are not easily obtained, but the approximate lethal dose is 0.1 mg. copper for a 10 gm. mouse.

To compare the local toxic actions of copper sulphate and the copper amino acid solution, the method of intracutaneous inoculation (using 1 c.c. tuberculin syringe, graduate in 0.01 c.c.) was used. Guinea-pigs were given intracutaneous injections of 0.2 c.c. of varying concentrations of the two copper compounds (1.0, 0.1, 0.01, and 0.001 percent copper concentration), one on one side of the back and the other on the opposite side, equal concentrations opposite each other. Daily observations were then made with results showing that the local toxicity of the copper amino acid mixture does not greatly differ from that of copper sulphate. The extent of the lesion produced is about the same in both cases; while the lesion is larger in the case of the former, it is deeper, as indicated by skin thickness, in the case of the latter. A difference, however, exists in the fact that the copper amino acid mixture produces a hemorrhagic lesion while the copper sulphate produces a simple necrosis in higher concentrations. A 0.01 percent copper concentration seems to be tolerated without a marked injury to the tissues, even tho 0.001 percent still causes a transient induration.

ABSORPTION OF COPPER BY INUNCTION

Absorption of copper by inunction in guinea-pigs was tried, but the results were unsatisfactory, as shown by the following experiments.

Two guinea-pigs received 2 gm. of 50 percent copper oleate (0.1 gm. copper) in lanolin, and four days later another 2 gm. Three days after the last inunction, the liver of Guinea-pig A was analyzed by the method given later in this paper, yielding 0.14 mg. copper in the total 13 gm. of liver. Eight days after the last inunction the total liver of Guinea-pig B, 13 gm., yielded 1.3 mg. copper.

A guinea-pig received 2 gm. of 50 percent copper oleate (0.1 gm. copper) in lanolin; four days later, 2 gm.; eight days later, 3 gm.; and seven days later 3 grams. The animal was dead on the ninth day after the last inunction. The total liver, 11 gm., yielded 0.3 mg. of copper.

A guinea-pig received (the solution was painted on and allowed to dry) 3 c.c. of copper amino acid solution (each cubic centimeter containing 15 mg. copper), and four days later another 3 c.c. Eight days after the last administration the total liver, 10 gm., yielded 0.21 mg. copper.

Result.—It can be stated, then, that copper is absorbed from the skins of guinea-pigs after administration of copper oleate in lanolin by inunction, or copper amino acid solutions painted on and allowed to dry, but the amounts absorbed are correspondingly small as compared to the amount administered, and that this method of administration offers many inconveniences as compared to other methods.

TABLE 1
THE EFFECT OF COPPER BY MOUTH ON TUBERCULOSIS OF THE RABBIT'S EYE
Series A. 0.01 mg. Human Tubercle Bacilli

No.	Time Copper Administered	Duration of Tuberculosis	Weight of Eye	
			Right	Left
2	109 days	Control	3.60	3.60
5	110 days	105 days	3.05	3.00
6	63 days	58 days
9	Control	107 days	3.85	2.95
10	Control	105 days	5.90	3.03

Series B. 0.05 mg. Human Tubercle Bacilli

3	78 days	Control	3.55	3.45
4	77 days	Control	3.10	3.10
7	77 days	72 days	5.15	2.60
8	77 days	72 days	6.30	3.00
11	Control	72 days	5.45	3.09
12	Control	73 days	5.50	2.70

THERAPEUTIC FEEDING EXPERIMENTS WITH COPPER SULPHATE ON NORMAL AND TUBERCULOUS RABBITS

Two series of six rabbits each were divided into three experiments: four of the rabbits merely serving as controls for the effect of the feeding of copper and to obtain the weights of normal eyes; four serving as infected controls receiving tubercle bacilli in the right eye

and not receiving copper, to watch the progress of the disease in the eye and to obtain the final weight of the infected eye after a definite period of time; and four serving as tests, being infected with tuberculosis in the eye and fed copper to determine whether or not the copper had any effect on the progress of the disease in the eye, as indicated by frequent observation of the process and the final weights of the unruptured eyes.

The copper was administered in the form of a 1 percent copper sulphate solution in 1-2 c.c. amounts, as indicated in Table 1. Briefly, it can be stated that the copper had no visible (macroscopical) effect upon the tissues of the control rabbits nor did it in any way affect the progress of the tuberculosis in the eyes of the copper-treated animals as compared to those of the animals not receiving copper. This was borne out by the final weights of the eyes as well as by the progress of the disease, as noted by frequent inspection.

THERAPEUTIC INJECTION EXPERIMENTS ON RABBITS

Four rabbits, as controls, were inoculated into the right eye with tubercle bacilli.

13.—A rabbit was inoculated into the anterior chamber of the right eye with a uniform suspension of 0.1 mg. human tubercle bacilli and met an accidental death 54 days after infection. There was a moderately advanced tuberculosis of the right eye but no pronounced enlargement of the eyeball.

14.—Duplicate of 13, infected for 71 days, revealed at this period a slight tuberculosis of the right eye resembling, in amount of tuberculosis, Rabbit 23. Killed by bleeding on the 321st day. Right eye (3.4 gm.) contained three small tubercles about 0.3 cm. in diameter; left eye (3.2 gm.) and the rest of organs were normal.

15.—Duplicate of 13, infected for 71 days and observed every few days, revealed at this time a well-advanced tuberculosis of the right eye. Died of snuffles on the 137th day. Right eye completely involved but not enlarged; right purulent pleuritis; left eye and the rest of the organs normal.

16.—Duplicate of 13, infected for 71 days, revealed a markedly advanced tuberculous right eye, bulging, and about to rupture. Died on the 132d day. Complete involvement of the right eye (2.5 gm.) which had ruptured. The liver was slightly fatty; pregnancy existed; lungs congested; the left eye (3.5 gm.) and rest of the organs were normal.

In the following therapeutic experiments copper sulphate, copper acetate, copper oleate, and a copper amino acid mixture were injected intramuscularly into rabbits eleven days after inoculation into the anterior chamber of the right eye with 0.1 mg. human bacilli, and before visible tuberculosis had developed in the eye.

17.—A rabbit was given 1 percent copper sulphate on alternate days from the 11th to the 48th days in amounts of 0.5-1.0 c.c., making a total of 34.4 mg. copper. On the 49th day the animal was thin, the right leg had developed an ulcer,

and there was a well-advanced tuberculosis in eye. Died on the 64th day. Right eye (4.0 gm.) tuberculous; the left eye normal (3.0 gm.); the liver contained numerous small necrotic areas and was pale; the kidneys were pale; the rest of the organs were normal.

18.—Was given 1 percent copper sulphate on alternate days from the 11th to the 46th days in amounts of 0.5-1.0 c.c., making a total of 31.8 mg. copper. In spite of the large amount of copper sulphate given, the tubercles in the eye gradually developed and did not differ from those in Controls 15 and 16. Died on the 49th day after infection. There was a moderate tuberculosis of the right eye (2.0 gm.), at this period identical with that found in Controls 15 and 16; normal left eye (2.0 gm.); necrosis in right leg; liver pale with a few small necrotic areas; rest of organs normal.

19.—Was given 1 percent copper acetate on the same days and in the same dosage as Rabbit 17, making a total of 42.9 mg. copper within a period of 37 days, at the end of which time injections were stopped because animal was failing. The development of tuberculosis in the right eye was about the same as that in control 15. On the 71st day after infection there was a well-advanced tuberculosis of the right eye. Bled to death on the 285th day. Tuberculosis of the right eye (5.0 gm.); the left eye (4.0 gm.) and other organs were normal.

20.—Duplicate of 19. The development of tuberculosis in the right eye was a little more rapid than in Control 16, and the eye ruptured on the 71st day. Bled to death on the 289th day. Well-advanced tuberculosis of the right eye (3.0 gm.); left eye (4.0 gm.) and rest of organs normal.

21.—Given 1 percent copper oleate in oil on alternate days from the 11th to the 48th days in amounts of 0.5-1.0 c.c., a total of about 13.0 mg. copper. The development of the tuberculosis in the right eye was not as rapid as in Control 15, but more rapid than in 14. On the 71st day there was a moderately advanced tuberculosis of the right eye, which on the 93d day involved the entire bulb, without enlargement. On the 304th day killed on bleeding. There was a completely involved (not enlarged) right eye (4.0 gm.); the left eye (4.0 gm.) and other organs normal.

22.—Given 1 percent copper oleate in oil on alternate days from the 11th to the 46th days in amounts of 0.5-1.0 c.c., a total of about 12.0 mg. copper. Died on the 49th day. The tuberculosis of the right eye had progressed about as much as in Controls 15 and 16; there was moderate tuberculosis of the right eye (3.0 gm.); a normal left eye (2.5 gm.); hemorrhagic lungs; a pale liver; other organs normal.

23.—Was given a solution of copper amino acid mixture, containing about 1.9 mg. copper and 1.8 mg. nitrogen per c.c., on alternate days from the 11th to the 48th days, in amounts of 0.3-1.0 c.c., a total of about 22.7 mg. copper. On the 71st day there was a moderate tuberculosis of the right eye which closely resembled that of Control 14. On the 133rd day there were numerous miliary tubercles on the iris, the anterior chamber contained pus, but the bulb was not enlarged. Died on the 244th day. Postmortem: The right eye was about the same as on the previous date; left eye normal; lungs hemorrhagic with numerous small foci of necrosis; the rest of the organs normal.

24.—Duplicate of 23, given a total of about 21.85 mg. copper. The development of the tuberculosis of the right eye was about as rapid as in Control 15. On the 71st day after infection there was a markedly advanced tuberculosis of the right eye and the process progressed as rapidly as in the controls. On the 133d day the entire bulb of the right eye involved but not ruptured, and not much enlarged. Killed on the 317th day. Right eye (2.1 gm.) ruptured and entirely involved with tuberculosis; left eye (3.0 gm.) and other organs normal.

As a result of these experiments the following conclusions seem justified: Copper sulphate, copper acetate, copper oleate, and copper amino acid mixture injected in rabbits intramuscularly in doses totaling 12.0-42.9 mg. copper during a period of thirty-seven days, the injections having been begun eleven days after intraocular infection with tubercle bacilli, and before visible development of tuberculosis, do not materially affect the progress of the tuberculous process in the eye during the period of injection, or for a period up to 269 days after the injections are stopped, even tho the doses of the various salts border on the toxic limit.

THE THERAPEUTIC INJECTION OF VARIOUS COPPER SALTS INTO GUINEA-PIGS AFTER INFECTION WITH TUBERCULOSIS

The weights of the guinea-pigs in the following series were taken frequently during the period of observation, but were found of no especial significance, hence are omitted.

Three guinea-pigs, as controls, were inoculated with tuberculosis and were not given copper.

25.—Female guinea-pig inoculated subcutaneously on the left side of anterior abdominal wall with bacilli, 0.1 mg. human bacilli. The development of the local tubercle was compared with that in copper treated animals. On the 70th day there was a large tuberculous nodule in the left mammary gland and the local lymph glands were about 2 cm. in diameter. Died on the 119th day. Post-mortem: The local glands were 1-2 cm. diameter; no enlarged retroperitoneal glands; peribronchial glands moderately enlarged; large necrotic areas involving nearly the entire liver; spleen enlarged and partly necrotic; lungs consolidated; pregnancy.

26.—Duplicate of 25. On 70th day after infection fairly large local tuberculous glands and a hard mass in the mid-line. Died on the 140th day. Post-mortem: The peribronchial and retroperitoneal glands were enlarged; spleen and liver contained numerous necrotic areas; lungs small foci of necrosis; hemorrhage into the peritoneal cavity.

27.—Duplicate of 25. On 70th day a fairly well-advanced local tuberculous mass of glands. Died on 159th day. Postmortem: The local, peribronchial, and retroperitoneal glands much enlarged; liver large masses of necrosis; spleen markedly enlarged with one large mass of necrosis; lungs multiple foci of necrosis; hemorrhagic fluid in the peritoneal cavity.

Four guinea-pigs were inoculated with tubercle bacilli in the same way as 25, 26, and 27, and were given copper in various forms.

28.—Female guinea-pig was inoculated with 0.1 mg. human bacilli and on the 11th day treatment was begun by intramuscular injection of 1 percent copper sulphate solution on alternate days to the 45th day, in amounts of 0.3-0.75 c.c., making a total of about 17.7 mg. copper. The tuberculosis in this guinea-pig progressed about equal to that of Control 25, which also gave birth to two

young. On the 70th day after infection the local glands measured about 2 cm. As a whole this animal compared well with Controls 25 and 26. Died on the 89th day. Postmortem: The local glands were large; spleen large with necrotic areas (7.0 gm.); liver pale with large foci of necrosis; lungs small foci of necrosis.

29.—Guinea-pig infected in the same way. From the 11th day copper acetate (1 percent solution) was given by intramuscular injection, and in the same doses as copper sulphate was given to Pig 28, making a total of 22.1 mg. copper. The local tuberculosis advanced more rapidly than in Controls 26 and 27. Local glands ruptured on the 59th day. Died on the 111th day. Postmortem: The local glands were small, peribronchial glands enlarged and hard; hemorrhagic peritonitis; liver and spleen enlarged with numerous large foci of necrosis; lungs numerous foci of necrosis.

30.—Guinea-pig infected in the same way. From the 11th day after infection, copper oleate (1 percent solution in oil) was given, by intramuscular injection on alternate days to the 45th day in amounts of 0.3-0.75 c.c., making a total of about 6.6 mg. copper. The tuberculosis in the local glands progressed about as in 26 and 27, and on the 70th day were about 2 cm. in diameter. Died on the 135th day. There was an ulcer at site of injection; local glands enlarged, peribronchial and retroperitoneal glands slightly enlarged; numerous large necrotic areas in the liver and spleen; multiple necrotic areas in lungs.

31.—Guinea-pig infected like the others. From the 11th day it received a copper amino acid mixture (containing 1.9 mg. copper and 1.8 mg. nitrogen per c.c.) intramuscularly on alternate days to the 45th day in amounts of 0.1-0.75 c.c., making a total of about 10.9 mg. copper. The local glands were only slightly enlarged on the 70th day after infection. Died on the 157th day. Postmortem: The local glands, retroperitoneal, and peribronchial glands enlarged; spleen markedly enlarged and made up of large necrotic areas; liver almost entirely necrotic; numerous large foci of necrosis in lungs, a few small areas in kidneys.

As a result of the experiments the following conclusion seems justified: Copper sulphate, acetate, oleate, and copper amino acids do not appreciably affect the progress of tuberculosis in guinea-pigs when given by injection on the eleventh day after infection, and continued for thirty-four days (a total of 6.6-22.1 mg. copper being given), as indicated by the duration of life and the lesions.

32.—Guinea-pig was inoculated subcutaneously on the left side of the anterior abdominal wall with 0.05 mg. human bacilli. The development of the local tubercle was carefully observed and compared with the developments in copper treated guinea-pigs. On the 52nd day small tuberculous nodules were present. Died on 180th day. Postmortem: The local, peribronchial, and retroperitoneal glands moderately enlarged; spleen about twice normal size, and full of small necrotic areas; a few necrotic areas in the liver; numerous necrotic areas in the lungs.

33.—Duplicate of 32; inoculated in same way. On the 52nd day after injection small local tuberculous glands were present; died on 89th day. Postmortem: The local glands were not much enlarged; spleen enlarged with large necrotic areas; liver and lungs full of necrotic areas; hemorrhage in the peritoneal cavity; tubercles in the omentum.

Eight guinea-pigs were inoculated with the same tubercle bacilli as 32 and 33 and received copper in various forms.

34.—Inoculated with 0.05 mg. human bacilli, and, on the 14th day, treatment was begun by intramuscular injection of a 1 percent copper sulphate solution as follows: 14th, 16th, and 19th days—0.75 c.c. each; 27th day—0.5 c.c.; a total of 7.0 mg. copper. On the 52nd day after injection, the tuberculosis in this animal had progressed just about as far as in the Controls 32 and 33. The copper injection produced necrosis and was stopped on the 27th day. Died on the 112th day. Local glands small; enlarged peribronchial lymph glands; large necrotic areas in the right lobe and numerous small such areas throughout the liver; spleen enlarged with numerous foci of necrosis; double hydrothorax; numerous small necrotic areas in lungs; kidneys pale.

35.—Duplicate of 34; on the 52nd day local glands larger than those of Controls 32 and 33. Died on the 182nd day. Local, retroperitoneal, and peribronchial glands enlarged; spleen markedly enlarged with large masses of necrosis; numerous large areas of necrosis in liver and lungs.

36.—Duplicate of 34 and 35; given intramuscular injections of a 1 percent copper acetate solution as follows: 14th, 16th, and 19th days;—0.75 c.c. each day; a total of 7.2 mg. copper. Died on 20th day after injection; postmortem revealed necrotic areas only at the site of injection. There was no visible enlargement of the local glands and the organs all appeared normal microscopically.

37.—Duplicate of 36; given copper acetate (1 percent solution) on the following day after injection; 14th, 16th, and 19th days; 0.75 c.c. each; 27th day, 0.5 c.c.; a total of 8.8 mg. copper. On the 52nd day after infection the size of the local glands was decidedly larger than that of the control. Died on the 96th day. Local glands about 1 cm. in diameter; peribronchial and retroperitoneal glands moderately enlarged; numerous foci of necrosis in the liver; spleen much enlarged and necrotic, adherent to the stomach; lungs numerous small foci of necrosis.

38.—Duplicate of 37; given intramuscular injection of 1 percent copper oleate in oil as follows: 14th, 17th, and 19th days—0.75 c.c.; 27th day—0.5 c.c.; a total of 2.7 mg. copper. On the 52nd day after infection there was a large hard mass in the region of the mammary gland, and the local lymph glands enlarged far more than those of the control. Died on the 119th day. Local, peribronchial, and retroperitoneal gland enlarged; liver necrotic; spleen enlarged with large foci of necrosis; numerous foci of necrosis in lungs.

39.—Duplicate of 38; given a total of 2.7 mg. copper. On the 52nd day after infection the local glands were distinctly palpable and at least as far advanced as the controls, which were not given copper. Died on the 183rd day. Local, retroperitoneal, and peribronchial glands enlarged; spleen enlarged and necrotic; numerous necrotic areas throughout the lungs.

40.—Duplicate of 39; received intramuscular injections of copper amino acid mixture containing 1.9 mg. copper and 1.8 mg. nitrogen per c.c. as follows: 14th, 16th, 19th, and 27th days, 0.5 c.c. each; a total of 3.8 mg. of copper. On the 52nd day after infection there was no palpable local lymph glands so that apparently, in this case, the local process was not marked. Died on the 250th day. Local, peribronchial, and retroperitoneal glands enlarged; liver full of necrotic areas; spleen enlarged and necrotic; numerous small necrotic areas in lungs.

41.—Duplicate of 40; given a total of 3.8 mg. copper. This guinea-pig died on the 33rd day after infection and revealed at postmortem no local tuberculous glands; numerous small miliary tubercles in the spleen; a few small necrotic areas in the liver; the rest of the organs normal. At the site of injections the muscles were necrotic.

As a result of these experiments it seems justified to state that copper, in the form of sulphate, acetate, oleate, and a copper amino acid mixture, in amounts totalling from 2.7 to 8.8 mg., had no appreciable effect on the progress of tuberculosis in guinea-pigs when begun fourteen days after infection and given for thirteen days, as indicated by the condition of the animals, the duration of life, and the lesions.

Guinea-pig 40 lived for 250 days, but this case is probably of no significance as shown by further experiments, in which copper amino acids were given to a large number of pigs for a much longer period of time and in larger amounts.

THE THERAPEUTIC INJECTION OF COPPER SALTS INTO GUINEA-PIGS BEGUN COINCIDENT WITH INFECTION WITH TUBERCULOSIS

Six guinea-pigs, as controls, were inoculated with tuberculosis and were not given copper.

42.—Inoculated subcutaneously on the left side of the anterior abdominal wall with 0.1 mg. human bacilli. Died on 94th day. Local and peribronchial glands enlarged; spleen enlarged and full of miliary tubercles; numerous small necrotic areas in liver and lungs; rest of organs normal.

43.—Duplicate of 42; died on the 118th day. Local, retroperitoneal, and peribronchial glands moderately enlarged; a few small indistinct necrotic areas in liver; one large necrotic mass in spleen.

44. Duplicate of 42; died on the 118th day. Local, retroperitoneal, and peribronchial glands moderately enlarged; spleen enlarged with numerous large areas of necrosis; lungs a few foci of necrosis; large foci of necrosis throughout liver.

45.—Duplicate of 42; died on the 87th day. Local, retroperitoneal, and peribronchial glands enlarged and hard; spleen enlarged and full of necrotic areas; a few necrotic areas in lungs.

46.—Duplicate of 42; killed on the 38th day. Local glands moderately enlarged; left retroperitoneal gland enlarged and caseous; peribronchial glands not appreciably enlarged; small miliary tubercles in spleen and liver; lungs normal.

47.—Duplicate of 42; killed on 38th day. Local glands moderately enlarged; left retroperitoneal gland enlarged and caseous; peribronchial glands slightly enlarged; spleen slightly enlarged with numerous miliary necrotic areas; a few miliary necrotic areas in liver and lungs.

Ten guinea-pigs were inoculated with tubercle bacilli with the same emulsion as the previous six controls, in the same dosage, and on the

same days. They were then given copper in the form of copper amino acids prepared from egg albumen.

48.—Was given copper injections intramuscularly for 3 days previous to infection and on alternate days to the 29th day, in 0.3 c.c. amounts, a total of 5.7 mg. copper. Died on the 32nd day after beginning copper treatment. Post-mortem: The local lymph glands enlarged; retroperitoneal glands slightly enlarged; several necrotic areas in the liver; spleen not much enlarged but full of miliary tubercles.

49.—Duplicate of 48, receiving copper on alternate days to the 31st day in 0.3 c.c. amounts, a total of 6.1 mg. copper. Killed by ether on the 120th day. Local and retroperitoneal glands moderately enlarged, peribronchial glands markedly so; spleen only slightly enlarged; a few necrotic areas in the lungs and liver.

50.—Duplicate of 49; died on the 181st day. Local and peribronchial glands slightly enlarged, retroperitoneal glands markedly so; spleen one large mass of necrotic tissue and much enlarged; liver full of large areas of necrosis; lungs full of small necrotic areas.

51.—Duplicate of 48; died on the 41st day. Local and left retroperitoneal glands enlarged; peribronchial glands slightly enlarged and hard; enlarged spleen with numerous pinhead sized necrotic areas; liver a few necrotic areas.

52.—Duplicate of 48; died on the 34th day. Enlarged retroperitoneal and local glands; spleen contained multiple miliary necrotic areas, no visible necrosis in liver.

53.—Duplicate of 49; died on 90th day. Local glands markedly enlarged; peribronchial glands hard and enlarged; liver numerous small areas of necrosis; spleen enlarged and full of necrotic areas; lungs numerous pinhead size necrotic areas.

54.—Duplicate of 48; died on 37th day. Local glands slightly enlarged; retroperitoneal glands not visibly enlarged; peribronchial glands enlarged and hard; spleen enlarged and full of necrotic areas of pinhead size; liver contained numerous miliary necrotic areas.

55.—Duplicate of 49; killed by ether on the 120th day. Local glands markedly enlarged; peribronchial glands moderately enlarged; spleen contained numerous necrotic areas and moderately enlarged; lungs full of small necrotic areas; a few such in liver.

56.—Duplicate of 49; died on the 93rd day. Local glands enlarged; peribronchial glands markedly enlarged; spleen markedly enlarged and necrotic; multiple large foci of necrosis in liver and numerous small foci in lungs.

57.—Duplicate of 48; died on the 34th day. Local and retroperitoneal glands slightly enlarged; peribronchial glands moderately enlarged; a few necrotic areas in the liver; spleen enlarged to twice its normal size and contained large foci of necrosis.

As a result of these experiments it seems justified to state that copper, in the form of copper amino acids, given to guinea-pigs two days previous to infection with tubercle bacilli and continued for twenty-nine to thirty-one days (totalling 5.7-6.1 mg. copper) did not appreciably affect the progress of the disease, as indicated by the condition, duration of life, and character of lesions.

THE THERAPEUTIC INJECTION OF COPPER SALTS IN GUINEA-PIGS PREVIOUS TO INFECTION WITH TUBERCULOSIS

Seven guinea-pigs, as controls, were inoculated with tuberculosis, but not given copper.

58.—Inoculated with a uniform emulsion of tubercle bacilli on the 19th day of observation; found dead on the 62nd day. The inguinal, retroperitoneal, and peribronchial glands enlarged and caseous; liver filled with small areas of necrosis; spleen large and soft; lungs full of necrotic areas, and a mass of adhesions and caseous substance in thorax.

59.—Killed by chloroform on the 88th day of observation. Enlarged and caseous glands in both groins; liver and spleen almost completely necrotic.

60.—Killed by chloroform on the 88th day. Large ulcer in the left groin; enlarged caseous glands in both groins; retroperitoneal and peribronchial glands enlarged; liver engorged with blood and contained a few miliary necrotic areas; spleen enlarged and full of miliary necrotic areas.

61.—Killed by chloroform on 88th day. A nearly healed ulcer and enlarged caseous gland in the left groin; retroperitoneal and peribronchial glands enlarged; liver and spleen full of miliary necrotic areas; lungs filled with translucent necrotic areas with caseous centers.

62.—Killed by chloroform on 88th day. Glands in left groin large and caseous; peribronchial glands enlarged; liver engorged with blood and contained a few miliary necrotic areas; spleen full of miliary necrotic areas; lungs showed a few translucent necrotic areas.

63.—Killed by chloroform on 88th day. Enlarged and caseous glands in the left groin; peribronchial and retroperitoneal glands enlarged; liver, large and engorged with blood, contained a few miliary necrotic areas; lungs dotted with translucent necrotic areas.

64.—Killed by chloroform on 88th day. Enlarged caseous glands in the left groin; retroperitoneal glands slightly enlarged; a few miliary necrotic areas in the liver and lungs; spleen packed with necrotic areas.

Eleven guinea-pigs were inoculated on the same days and with similar doses of the same emulsion of tubercle bacilli as the previous seven controls; in addition they were given injections of copper amino acids on alternate days for nineteen days previous to and six days after infection.

65.—Given copper injections of a mixture containing 1.5 mg. copper per c.c. intramuscularly in amounts from 0.3 to 0.5 c.c. until 5.7 mg. copper had been given. Chloroformed on 95th day. A partly healed ulcer on the left side of the abdominal wall under which was a mass of caseous substance; peribronchial and retroperitoneal glands enlarged and caseous; liver full of miliary necrotic areas; spleen enlarged and filled with miliary necrotic areas, borders somewhat necrotic; lungs dotted with large fibrous tubercles.

66.—Chloroformed on 95th day. A group of large caseous glands in the left groin; anterior mediastinal, peribronchial, and retroperitoneal glands all enlarged and caseous; abdomen full of bloody fluid; firm adhesions around liver and spleen; liver and spleen dotted with miliary necrotic areas; lungs filled with large caseous and fibrous tubercles.

67.—Chloroformed on 95th day. Ulcer discharging caseous substance on left side of the abdominal wall; enlarged glands in both groins, peribronchial glands enlarged; liver dotted with yellowish-green necrotic areas; spleen large and filled with caseous necrotic areas; lungs filled with large fibrous tubercles.

68.—Died on 84th day. Enlarged caseous glands in both groins and axillae; retroperitoneal and peribronchial glands enlarged and caseous; liver pale, several small necrotic areas; both lungs hard and filled with necrotic areas.

69.—Died on 88th day. Enlarged caseous glands in both groins; liver contained several necrotic areas; spleen large and filled with necrotic areas; lungs filled with translucent tubercles having caseous centers.

70.—Died on 87th day. Ulcer in left groin, large caseous nodules in both groins; liver and spleen full of necrotic areas; lungs full of translucent tubercles.

71.—Died on 49th day. Glands in both groins enlarged and necrotic; liver, many miliary necrotic areas; spleen enlarged and filled with miliary necrotic areas; lungs not much involved—only a few small early tubercles.

72.—Died on the 49th day. Enlarged and caseous glands in both groins; liver, spleen, and lungs appear normal. Cause of death possibly post partum infection.

73.—Died on the 85th day. Partly healed ulcer in the left groin; enlarged and caseous glands in both groins; peribronchial glands enlarged; liver and spleen large and filled with caseous necrotic areas; lungs dotted with necrotic areas.

74.—Died on 50th day. Glands enlarged and caseous in both groins; spleen enlarged and full of miliary necrotic areas; liver contains a few small yellow areas of necrosis; lungs normal; pericardium filled with cloudy fluid.

75.—Died on the 77th day. A large mass of caseous substance in the abdominal wall; large caseous gland in left groin, enlarged but not caseous gland in right groin; peribronchial glands enlarged and caseous; two small necrotic areas in the liver; spleen full of miliary necrotic areas; lungs solid and filled with large necrotic areas.

As a result of these experiments the following conclusion seems justified: Administration of copper, in the form of copper amino acids, to guinea-pigs for nineteen days previous to and six days after infection with tubercle bacilli, totalling 5.7 mg. copper, had no appreciable effect upon the course of the tuberculosis, as indicated by the lesions. The majority, eight out of eleven, of the guinea-pigs treated with copper died within forty-nine to eighty-eight days, while only one control died in sixty-two days.

THE DISTRIBUTION OF COPPER IN THE TISSUES OF TUBERCULOUS ANIMALS

In the following analyses the colorimetric method for copper was chosen. Even tho colorimetric methods are never as accurate as gravimetric methods, they are frequently more delicate, as in the case of copper when dealing with small amounts, and at the same time they

give approximately accurate results for the larger amounts. We are more interested in determining the presence of small amounts of copper in these experiments than in determining with absolute accuracy the larger amounts.

Method.—The following method, as shown by controls with known amounts of copper added to tissues, proved to be quantitatively delicate down to 0.05 mg. copper, and qualitatively would still reveal amounts down to 0.01 mg. copper. A sample of 10-15 gm. of the tissue was weighed out in a large evaporating dish or Kjeldahl flask, and to it added 10 c.c. of chemically pure sulphuric acid. All reagents were tested for the presence of copper, and were only used when copper was not present in amounts determinable by the above method. When ready for analysis, 10-20 c.c. nitric acid were added and the mixture carefully heated to prevent loss by frothing. Frequent additions of small amounts of nitric acid were then made and the mixture carefully heated until all the organic matter had been destroyed. Finally, it was heated more strongly until the sulphuric acid was practically colorless, the addition of a few cubic centimeters more sulphuric acid being necessary at times. The residue was then washed carefully into a 100-150 c.c. Jena beaker by means of a small amount of distilled water (copper free—distilled in glass) and finally neutralized to litmus by means of a strong solution of sodium hydroxid (chemically pure and free from copper). After neutralization, 1-2 c.c. of sulphuric acid¹⁷ (concentrated sulphuric acid diluted with an equal volume of water), and sufficient distilled water was added to bring the volume to about 80 c.c. From this solution the copper was then separated by electrolysis, being kept covered during the entire process.¹⁸ A fine platinum wire spiral was used as depositing electrode, and a current of sufficient voltage and amperage to deposit quantitatively from 0.05 mg. copper upward within 7-8 hours, as shown by controls with known amounts of copper. After deposition was complete, the electrodes were carefully washed off by means of several changes of distilled water while the current was still on, or until stoppage of the current, as indicated by absence of gas bubbles on the electrodes, and finally by a single change of alcohol. The depositing electrode was then placed in a small Jena glass beaker and the alcohol evaporated off in a hot-air oven at 100° C. The copper was then dissolved off from the electrode with a few cubic centimeters of concentrated nitric acid heated on a water bath. This was then ready for colorimetric determination, comparison being made with a known standard solution of copper containing 0.1 mg. copper per cubic centimeter in a Du Bosc colorimeter. To obtain complete solution of the copper nitrate 0.2-0.4 c.c. of an acetic acid solution (glacial acetic acid diluted with 10 parts of distilled water) was added, equal amounts also being added to the known standard copper solution, and both known and unknown being diluted to 10 c.c. with distilled water (0.05-0.1 mg. copper were found to give the best results colorimetrically and a fraction of the unknown was used which could be compared with this). The amount of acetic acid in the known and unknown solutions compared must also be about equal. To these was then added 1 c.c. 50 percent ammonium nitrate and 1 c.c. of a 4 percent solution of potassium ferrocyanid (freshly prepared each time).¹⁹ Comparisons were then immediately made by placing the solutions in the chamber of the Du Bosc colorimeter, since changes in color soon occur.

17. Foerster: Berlin, 1906, 39, p. 3029.

18. Merick: Chem. News, 1876, 33, p. 111; Kohn: Jour. Soc. Chem. Industry, 1891, 10, p. 327.

19. Carnelley: Chem. News, 1875, 32, p. 308.

ANALYSES OF TISSUES OF GUINEA-PIGS

The number of Animals 25-76 correspond with those in previous series, so that the details as to lesions may be compared.

25.—Normal pig infected with tuberculosis; organs analyzed 119 days afterward. The lungs (9.0 gm. of a total 18.0 gm.), local glands (2.5 gm.), peribronchial glands (1.0 gm.), spleen (10.0 gm.), and kidneys (7.0 gm.) contained no copper. The liver (13.0 gm. samples) contained 0.185 mg. copper in the total 37 gm.

26.—Normal pig infected with tuberculosis; organs analyzed on the 140th day. The spleen (30.0 gm.) contained no copper, while the liver (total 40.0 gm.) contained a trace (in 20.0 gm. samples).

27.—Normal pig infected with tuberculosis; organs analyzed on the 159th day. The spleen (29.0 gm.) contained no copper, while the liver (total 64.0 gm.) contained a trace (in 11.0 gm.).

28.—Guinea-pig infected with tuberculosis received intramuscularly 1 percent copper sulphate solution, a total of 17.7 mg. copper; died 44 days after the last injection. The local glands (2.5 gm.), mammary pus (2.5 gm.), and spleen (7.0 gm.) contained no copper. The liver (12.0 gm. samples) contained 0.007 mg. copper per 1 gram tissue.

29.—Infected with tuberculosis and given intramuscularly 1 percent copper acetate solution, a total of 22.1 mg. copper; died 66 days after the last injection. The local (2.0 gm.) and peribronchial lymph glands (1.5 gm.), spleen (9.0 gm.), and lungs (12.0 gm.) contained no copper. The kidneys (9.0 gm.) contained 0.063 mg. and the liver (11.0 gm. samples), 0.368 mg. in the total 46.0 gm.

30.—Infected with tuberculosis and given intramuscularly 1 percent copper oleate in oil, a total of about 6.6 mg. copper; died 90 days after the last injection. The spleen (8.0 gm.) contained a trace, and the liver (10.0 gm. samples), 0.40 mg. in the total 50.0 gm.

33.—Normal pig was infected with tuberculosis and on the 89th day its organs were analyzed. The spleen (8.0 gm.) and the kidneys (8.0 gm.) contained no copper. The liver contained 0.12 mg. in the total 20.0 gm.

34.—Infected with tuberculosis and given intramuscular injections of 1 percent copper sulphate solution, a total of 7.0 mg. copper. Died on the 85th day after the last injection. The liver (40.0 gm.) contained no copper in 20.0 gm. samples.

37.—Infected with tuberculosis and received intramuscular injections of 1 percent copper acetate solutions, a total of 8.8 mg. copper. Died on the 69th day after the last injection. The lungs (6.0 gm.), spleen (8.0 gm.), and kidneys (6.0 gm.) contained no copper. The liver (8.0 gm. sample) contained 0.019 mg. copper per 1 gram tissue.

38.—Infected with tuberculosis, given intramuscular injections of 1 percent copper oleate in oil, a total of 2.7 mg. copper. Died on 92nd day after the last injection. The liver (35.0 gm.) contained no copper in 10 gm. samples.

42.—Normal pig, infected with tuberculosis; on the 94th day after infection its liver (18.0 gm.) was analyzed and contained no copper in 10.0 gm. samples.

43.—Normal pig, infected with tuberculosis; on the 118th day after infection its liver (10.0 gm. samples) was analyzed and contained 0.204 mg. copper in the total 34.0 gm.

44.—Normal pig, infected with tuberculosis; on the 118th day after infection its liver (10.0 gm. samples) was analyzed and contained 0.145 mg. copper in the total 29.0 gm.

45.—Normal pig, infected with tuberculosis; on the 87th day after infection its organs were analyzed. The local glands (3.0 gm.), spleen (10.0 gm.), kidneys (5.0 gm.), and liver (11.0 gm. samples of total 24.0 gm.) contained no copper.

46.—Normal pig, infected with tuberculosis; on the 38th day after infection its liver contained 0.090 mg. copper in the total 18.0 gm.

47.—Normal pig, infected with tuberculosis; killed on the 38th day after infection and its liver contained 0.072 mg. copper in the total 18.0 gm.

48.—Given copper (copper amino acids) on alternate days for 29 days, a total of 5.7 mg. copper, and on the 3d day was infected with tuberculosis. Died three days after the last copper injection. The liver (10.0 gm. samples) contained 0.928 mg. copper in the total 16.0 gm.; the bile (1 c.c.) contained no copper.

49.—Given copper (copper amino acids) on alternate days for 31 days, a total of 6.2 mg. copper, and on the third day was infected with tuberculosis. Killed on the 89th day after the last copper injection. Local, peribronchial, and retroperitoneal glands (7.0 gm.) contained no copper; the liver (11.0 gm. samples) contained 0.180 mg. in the total 30.0 gm.

51.—Duplicate of 48; died on the 12th day after the last copper injection. The liver and gall bladder contained 0.24 mg. copper in the total 12.0 gm.

52.—Duplicate of 48; died on the 5th day after the last injection. The liver (11.0 gm. sample) contained 0.770 mg. copper in the total 14.0 gm.

53.—Duplicate of 49; died 51 days after the last injection. Kidneys (4.0 gm.) and spleen (4.0 gm.) contained no copper. The liver contained 0.105 mg. copper in the total 15.0 gm.

54.—Duplicate of 48; died on the 8th day after the last injection. The liver contained 0.590 mg. copper in the total 10.0 gm.

55.—Duplicate of 49; killed on the 89th day after the last injection. Local, peribronchial, and retroperitoneal glands (4.0 gm.) contained no copper; the liver (10.0 gm. samples) contained 0.36 mg. copper in the total 24.0 gm.

57.—Duplicate of 48; died on the 5th day after the last injection. The liver (11.0 gm.) contained 0.270 mg. copper in the total 15.0 gm.

76.—Infected with tuberculosis and 68 days later given intramuscular injections of copper amino acids in 0.3 c.c. amounts on alternate days for 58 days, a total of 11.1 mg. copper. Killed on the second day after the last injection. The local and peribronchial glands (3.0 gm.) and lungs (11.0 gm.) contained no copper; the kidneys contained 0.09 mg. in a total 5.0 gm.; blood (7.0 gm. sample) 0.003 mg. per 1 gm.; and the liver 1.819 mg. copper in a total 17.0 gm.

Local, peribronchial, and retroperitoneal glands enlarged; miliary tuberculosis in spleen; a few miliary necrotic areas in liver; lungs numerous large necrotic areas and consolidation.

77.—Infected with tuberculosis and 68 days later given intramuscular injections of copper amino acid mixture in 0.3 c.c. amounts on alternate days for 31 days, a total 6.15 mg. copper. Died on the third day after the last injection. The local glands (3.0 gm.) contained no copper; the spleen (5.0 gm.) a questionable trace; the kidneys (6.0 gm.) a trace; and the liver (10.0 gm. samples) 2.17 mg. copper in the total 25.0 gm.

Postmortem: Local, retroperitoneal, and peribronchial glands were markedly enlarged; spleen enlarged and full of necrotic areas; large foci of necrosis in liver; small foci of necrosis in lungs.

78.—Infected with tuberculosis and 68 days later given intramuscular injections of copper amino acid mixture in 0.3 c.c. amounts on alternate days for 52 days, a total of 10.2 mg. copper. Died on the 3rd day after the last injection. The local glands (5.0 gm.) contained no copper, the liver (12.0 gm. samples) 2.290 mg. in the total 24.0 gm. Postmortem: The local glands were markedly enlarged; peribronchial glands moderately enlarged; spleen enlarged and full of necrotic areas; liver contained isolated large foci of necrosis; lungs numerous small foci of necrosis.

79.—Infected with tuberculosis and 63 days later given 2 intramuscular injections, 2 days apart, of copper amino acid mixture, a total of 0.5 c.c. (0.75 mg. copper); died the day after the last injection. The liver contained 0.184 mg. copper in the total 23.0 gm. Postmortem: The local glands were markedly enlarged; spleen enlarged and necrotic; numerous small foci of necrosis in lungs; large foci in liver.

80.—Infected with tuberculosis and 61 days later given intramuscular injections of copper amino acid mixture on alternate days for 87 days, a total of 16.05 mg. copper. Died on the 2nd day after the last injection. The lungs (7.0 gm.) contained no copper; kidneys (6.0 gm.) a definite trace; and the liver (10.0 gm. samples) 0.736 mg. in the total 16.0 gm.

Postmortem: The local glands were moderately enlarged; spleen enlarged and contained necrotic areas; liver contained a few miliary necrotic areas.

81.—Infected with tuberculosis and 57 days later given intramuscular injections of copper amino acid mixture in 0.3 c.c. amounts on alternate days for 52 days, a total of 10.2 mg. copper. Died on the 3rd day after the last injection. The peribronchial and local glands (2.0 gm.) contained no copper; kidneys (5 gm.) 0.155 mg.; and the liver 3.297 mg. in the total 21.0 gm.

Postmortem: The local glands enlarged and full of pus; peribronchial and retroperitoneal glands hard and enlarged; spleen enlarged three times normal size, large foci of necrosis; large foci of necrosis in liver; multiple foci of necrosis 1 mm. in diameter in lungs.

82.—Infected with tuberculosis and 59 days later given intramuscular injections of copper amino acid mixture in 0.3 c.c. amounts on alternate days for 101 days, a total of 19.2 mg. copper. Died on the 3rd day after the last injection. The consolidated lungs (10.0 gm.) contained no copper, and the liver 0.198 mg. in the total 11.0 gm.

Postmortem: The local glands moderately enlarged; spleen twice normal size and full of small necrotic areas; lungs contained numerous necrotic areas and consolidated; liver contained a few small necrotic areas.

83.—Infected with tuberculosis and 40 days later given intramuscular injections of copper amino acid mixture in 0.3 c.c. amounts on alternate days for 29 days, a total of 5.7 mg. copper. Died on the 2nd day after the last injection. Local glands (2.0 gm.) contained no copper; the spleen (8.0 gm.) a questionable trace; the kidneys 0.147 mg. in a total 7.0 gm.; and the liver (10.0 gm. samples) 2.232 mg. in the total 36.0 gm.

Postmortem: The local, peribronchial, and retroperitoneal glands enlarged; multiple small necrotic areas in the lungs; spleen enlarged and necrotic; liver practically one-half necrotic.

84.—Normal guinea-pig, received copper for 39 days on alternate days in the form of copper amino acid mixture given intramuscularly in 0.3 c.c. amounts, making a total of 7.65 mg. Died on the 3rd day after the last injection. The liver contained 0.630 mg. copper in the total 10.0 gm.

85.—Guinea-pig. Received intramuscular injections of copper amino acid mixture in 0.3 c.c. amounts on alternate days for 49 days and a final 0.3 c.c. on the 54th day, a total of 9.0 mg. copper. The animal was killed 57 days after the last injection. The liver contained 0.608 mg. copper in the total 19.0 gm.

ANALYSES OF TISSUES OF RABBITS

17.—A rabbit was infected in the right eye and 11 days later intramuscular injections of 1 percent copper sulphate given on alternate days for 37 days, a total of 34.4 mg. copper. Died 16 days after the last injection. The tuberculous right eye (4.0 gm.) and normal left eye (3.0 gm.) contained no copper; the lungs contained 0.112 mg. copper in a total 14.0 gm.; the kidneys 0.126 mg. in a total 14.0 gm.; and the liver (20.0 gm. samples) 0.210 mg. per 1 gm. tissue.

15.—Rabbit infected in the right eye; received no copper. Died on the 137th day after infection and the liver (20.0 gm. sample) contained 0.065 mg. copper in a total 65.0 gm.

20.—Infected in the right eye; received no copper. Died on the 132nd day after infection. The normal left eye (3.5 gm.) and the tuberculous right eye (2.5 gm.), the lungs (14.0 gm.), kidneys (19.0 gm.), and liver (18 gm. samples of a total 83.0 gm.) contained no copper.

23.—Infected in the right eye and given intramuscular injections of copper amino acid mixture, a total of about 22.7 mg. copper. Died 196 days after last injection. The liver (15.0 and 16.0 samples) contained 0.001 mg. copper per 1 gram tissue.

18.—Infected in the right eye and given intramuscular injections of 1 percent copper sulphate, a total of 31.8 mg. copper. Died on the 3rd day after last injection. The tuberculous right eye (2.0 gm.), normal left eye (2.0 gm.), and lungs (6.0 gm.) contained no copper. The left kidney contained 0.065 mg. in a total 5.0 gm.; right kidney 0.06 mg. in a total 5.0 gm.; and the liver (9.0 gm. samples) 0.274 mg. per 1 gm. tissue.

22.—Infected in the right eye and given intramuscular injections of 1 percent copper oleate in oil, a total of about 12.0 mg. copper. Died 3 days after the last injection. The tuberculous right eye (3.0 gm.), normal left eye (2.5 gm.), and lungs (13.0 gm.) contained no copper. The right kidney contained 0.060 mg. copper in a total 6.0 gm.; left kidney 0.098 mg. in the total 7.0 gm.; and the liver (10.0 gm. sample) 0.041 mg. per 1 gram tissue.

86.—Non-infected rabbit received copper per os daily for 17 days in amounts varying from 2-4 c.c. of copper amino acid solution containing 15.0 mg. copper per cubic centimeter until a total of 600 mg. copper had been given. The animal died the day following the last feeding. Both eyes (8.0 gm.) contained no copper; lungs 0.034 mg. in the total 17.0 gm.; kidneys 0.180 mg. in a total 20.0 gm.; and liver (19.0 and 13.0 gm. samples) 0.050 mg. per 1 gram tissue. Postmortem: The eyes normal; kidneys normal; hemorrhagic fluid in the peritoneal cavity; lungs hemorrhagic and liver fatty.

87.—Non-infected rabbit received copper per os daily for 14 days in amounts varying from 1-4 c.c. of 10 percent copper oleate in peanut oil, a total of 330 mg. copper. Etherized the day after the last feeding. Right eye (3.0 gm.), left

eye (4.0 gm.), lungs (10.0 gm.), and right kidney (7.0 gm.) contained no copper; the liver (18.0 and 20.0 gm., samples) 0.001 mg. per 1 gm. tissue.

88.—Infected in the right eye with 0.1 mg. human tubercle bacilli and 73 days later feeding begun of copper amino acid solution containing 15.0 mg. copper per cubic centimeter daily 1-2 c.c. for 15 days, a total of 360 mg. copper. Died the day after last feeding. The tuberculous right eye (4.0 gm.) and normal left eye (4.0 gm.) contained no copper, the lungs (25.0 gm. sample) contained 0.078 mg. in the total 39.0 gm.; kidneys 0.087 mg. in total 17.5 gm. and the liver (19 gm. samples) 4.900 mg. in the total 98.0 gm.

Postmortem: The right eye, well advanced tuberculosis; left eye, liver, kidneys, and spleen normal; lungs marked fibrinous pleurisy.

89.—Infected in the right eye and given copper in the same form as 88, starting 73 days after infection, 1-2 c.c. daily for 45 days, a total of 735 mg. copper. Killed the day after the last feeding. The tuberculous right eye (5.0 gm.) normal left eye (4.0 gm.), lungs (8.0 gm.), and mammary gland (24.0 gm. sample) contained no copper; the blood (18.0 gm. sample) contained a questionable trace; and the kidneys 0.014 mg. in the total 14.0 gm.

Postmortem: There was well-developed tuberculosis of the right eye; left eye, liver, spleen and kidneys appeared normal; lungs, a few small necrotic areas.

90.—Infected in the right eye and given copper in the same form as 88, starting 73 days after infection, a total of 1,380 mg. copper in 97 days. Killed by bleeding on the second day after the last feeding. The tuberculous right eye (4.0 gm.) and normal left eye (4.0 gm.) contained no copper; lungs (11.0 gm.) contained a questionable trace; blood (10.0 gm. sample) 0.008 mg. per 1 gram tissue; kidneys (7.0 gm. sample) 0.165 mg. copper in the total 15.0 gm.; and the liver (11.0 and 15.0 gm. samples) 6.954 mg. in the total 61.0 gm.

Postmortem: Right eye well-advanced tuberculosis, but not enlarged; left eye and the rest of the organs appeared normal.

91.—Infected in the right eye, and 102 days later intramuscular injection begun into the hind legs on alternate days of copper amino acid solution, containing 1.5 mg. copper per cubic centimeter, 0.5-1.0 c.c. for 90 days, a total of 55.1 mg. copper. The animal died on the 3rd day after the last injection. The tuberculous right eye (5.0 gm.) and normal left eye (3.0 gm.) contained no copper; the right lung (total lungs 40.0 gm.) contained a questionable trace of copper in 15.0 gm., normal left lung (9.0 gm.) a trace; the heart (13.0 gm.) a trace; the right (8.0 gm.) and left (8.0) kidneys a trace; and the liver (10.0 and 13.0 gm. samples) 16.641 mg. copper in the total 83.0 gm.

Postmortem: Well-advanced tuberculosis of the right eye; left eye, liver, spleen and kidneys normal; right lung consolidated and containing small necrotic areas (snuffles); left lung a few hemorrhagic areas.

92.—Infected in the right eye and 102 days later received copper in the same form as Rabbit 91, a total of 23.6 mg. copper in 38 days. Killed by bleeding on the third day after the last injection. The tuberculous right eye (3.0 gm.), normal left eye (2.5 gm.) and lungs (7.0 gm. sample) contained no copper; the blood (19.0 gm. sample) contained 0.004 mg. copper per 1 gram tissue; kidneys 0.066 mg. in the total 11.0 gm.; and liver (11.0 gm. samples) 19.778 mg. in total 44.0 gm.

Postmortem: Right eye about normal size but entirely tuberculous, left eye normal; rest of organs normal.

93.—Given intramuscular injections of copper as follows: into the right thigh—copper amino acid solution 3 c.c. containing 12.0 mg. copper, and in the left thigh—1 percent solution copper sulphate, 3 c.c. containing 7.5 mg. copper; in five hours the animal was killed. The liver (30.0 gm. samples) contained 1.494 mg. copper in total 83.0 gm., and the blood (17.0 gm. sample) 0.007 mg. per 1 gram tissue.

As a result of the analyses the following conclusions seem justified:

1. Copper, in the form of copper amino acid mixture, injected intramuscularly into tuberculous guinea-pigs for a long period of time (2-100 days), enters the animal organism and is found within a few days after administration in largest amount and mainly in the liver, in small amounts in the kidneys and in traces in the blood and spleen, but never in appreciable amounts in the tuberculous lesions (lymph glands or pus), that is, less than 0.01-0.05 mg. in 3 gm. of material.

2. The copper in the liver gradually disappears, but remains in fairly large amounts up to the twelfth day, great variation being found in different individuals.

3. Copper, in the form of the copper amino acid mixture fed to normal and tuberculous rabbits for a long period of time (15-97 days), enters the animal organism and is found within a few days after administration mainly and in largest amounts in the liver, in small amounts in the kidneys and in traces in the blood and lungs, but not appreciably in either the normal or tuberculous eyes.

These results are in agreement with those of Chittenden,²⁰ who observed, by gravimetric method, that copper fed to dogs was found in greatest amount in the liver, in small, but determinable, amounts in the kidneys and none in the stomach, small intestine, heart, lungs, brain, spinal cord, spleen or muscle. Copper was never demonstrated in the urine, so that Chittenden concludes it is eliminated with the feces by way of the bile. They also agree with the results of Titze and Wedemann²¹ who analyzed the organs of two goats after having fed them copper sulphate and found the liver to contain the largest amount of copper, and the kidneys, spleen, heart and blood small amounts. The mammary glands, muscle and fatty tissues were free from it.

4. Copper, in the form of copper amino acid mixture, and as copper sulphate, injected intramuscularly into normal and tuberculous rabbits for a long period of time (37-90 days), enters the animal organism and is found within a few days after administration mainly and in largest

20. Rept. U. S. Dept. Agric., 1911-13, 97, p. 435.

21. Arb. a. d. k. Gsndhtsamte, 1911-12, 38, p. 125.

amounts in the liver, in small amounts in the kidneys, traces in the blood and lungs, but not appreciably in either the normal or tuberculous eyes.

5. Copper, in the form of copper sulphate, given intramuscularly for a long period of time (37 days), was still present in the liver in large amounts after sixteen days. Copper (as copper sulphate and copper amino acid mixture) given intramuscularly is present in the liver in fairly large amount (about 8 percent of amount given) after a period of five hours. A trace is also present in the blood at this time.

THE DISTRIBUTION OF COLLOIDAL COPPER IN TUBERCULOUS ANIMALS

In order to throw light on the mode of transportation of copper when injected or fed in simple inorganic salt form, it was thought advisable to compare its distribution in the animal organism under those conditions and when given intravenously in the colloidal form. Incidentally, these experiments would also throw light on the entrance of colloidal copper into the tubercle.

The colloidal copper used in these experiments was prepared by Bredig's method,²² using the electric arc, and the solutions were analyzed for their copper content colorimetrically.

94.—A rabbit (2100 gm.) was given 3 intravenous injections of 40 c.c. colloidal copper (80-90 mg. copper per liter). Died on fifth day. Both eyes (7.0 gm.) contained no copper; the heart (10.0 gm.) a questionable trace; both lungs (21.0 gm.) a trace; the left kidney, 0.117 mg. in the total 9.0 gm.; and the liver (10.0 and 13.0 gm. samples) 3.92 mg. in the total 80.0 gm. The liver had a peculiar mottled appearance; lungs, left kidney, spleen, and eyes appeared normal; right kidney, subcapsular hemorrhage.

95.—A rabbit (1850 gm.) with a tuberculous right eye was given colloidal copper (60-90 mg. copper per liter) intravenously, 10 c.c. daily for 12 days, a total of about 9.6 mg. copper. Died (of "snuffles") the day after the last injection. The tuberculous right eye (2.0 gm.), normal left eye (3.0 gm.), and heart (12.0 gm.) contained no copper. The consolidated right lung (total lungs 24.0 gm.) contained a trace in 14.0 gm.; the right (10.0 gm.) and left (10.0 gm.) kidneys a trace; and the liver (12.0 gm. samples) 2.32 mg. in a total 80.0 gm.

Postmortem: Right lung, consolidated; liver, kidneys, and left eye normal; right eye not enlarged but entirely tuberculous.

As a result of these analyses it is seen that copper administered to rabbits intravenously, in the form of colloidal copper, is distributed in the animal organism like the copper given intramuscularly in crystalloid form (copper sulphate and copper amino acid). The larger por-

tion found was present in the liver, small amounts in the kidneys, traces in the lungs and none in the eyes, normal or tuberculous.

These experiments, tho not entirely conclusive, would seem to indicate that copper is distributed throughout the animal organism after injection, not in simple salt form, but rather in the colloidal form. Since colloids do not readily enter tuberculous tissues, as was found to be the case by Wells and Helenburg,²³ who used, as colloid, egg albumin, we would not expect copper to enter these tissues, and this is borne out by the analyses. In addition, the fact that colloidal copper does not enter tuberculous tissues is in agreement with the results obtained with egg albumin, and simply adds another fact to bear out the statement made by Wells and Hedenburg, that colloids are not well suited as vehicles to carry chemical groups into tubercles.

GENERAL SUMMARY

Copper, in simple salt form (sulphate, acetate, oleate, and copper amino acid mixture prepared from hydrolyzed egg albumin), injected intramuscularly into normal and tuberculous guinea-pigs, in total amounts from 0.75 mg. up to about 19.0 mg. for about two to one hundred days, enters the animal organism and is found mainly and in largest amounts in the liver, in small amounts in the kidneys, in traces in the spleen, lungs, and blood, and not at all in the tuberculous lymph glands and pus. After cessation of injections the copper slowly but gradually decreases in amount in the liver.

Copper, in simple salt form, injected intramuscularly in guinea-pigs, in amounts varying from 2.7 to 22.1 mg., during a period of thirteen to thirty-four days, previous to (nineteen days), coincident with (two days before), or after (eleven days) infection with tubercle bacilli, has no appreciable effect upon the progress of the tuberculosis, as indicated by the lesions present after death.

Copper, in simple salt form, fed to rabbits in amounts up to 1,380 mg. of copper, during a period of ninety-seven days, and injected (copper sulphate and copper amino acids) intramuscularly into rabbits, in amounts up to 55.1 mg. of copper, during periods to ninety days, enters the blood and is found mainly and in largest amounts in the liver, in small amounts in the kidneys, in traces in the lungs and blood, and not at all in either the tuberculous or normal eyes.

23. *Jour. Infect. Dis.*, 1912, 11, p. 349.

Copper, in the form of copper sulphate, fed to rabbits in amounts of 1 to 2 c.c. of a 1 percent solution daily for up to one hundred days, begun five days before infection, has no appreciable effect upon the course of the tuberculosis of the eye, as indicated by the progress of the disease and the final weight of the eye.

Copper, in simple salt form, injected intramuscularly into rabbits in amounts of 12 to 42.9 mg. copper, for thirty-seven days, begun on the eleventh day after infection, has no appreciable effect upon the course of the tuberculosis of the eye, as indicated by the development of the disease in the eye.

Colloidal copper, prepared electrolytically by Bredig's method, injected intravenously into normal and tuberculous rabbits in amounts totalling 9.6 mg., from four to twelve days, is found mainly and in largest amounts in the liver, in smaller amounts in the kidneys, in traces in the lungs, and not at all in the normal or in the tuberculous eyes. This distribution of colloidal copper seems to favor the conception that copper in simple crystalline form, fed or injected intramuscularly into the animal organism, does not circulate in this simple form, but rather immediately forms colloidal combinations. If such is the case, this would readily explain its inability to enter tuberculous tissues, since it was found by Wells and Hedenburg that colloids (egg albumin) are not suited as entrants into the tubercle or other necrotic areas.

Copper, in the form of copper amino acid mixture, does not differ greatly in its systemic (tested in mice with the lethal dose intraperitoneally and intramuscularly about 0.05 to 0.1 mg. copper per mouse of 10 gm.) and local (tested intracutaneously in guinea-pigs) toxicity from that of copper sulphate when equal copper contents were compared. Locally, however, a difference existed in the fact that the copper amino acid mixture produced a hemorrhagic necrotic lesion, whereas the copper sulphate produced a simple necrosis. The local non-necrotizing concentration proved to be 0.01 percent copper, while 0.001 percent copper still produced a slight duration.

Copper, in the form of copper oleate (50 percent) in lanolin by inunction, and copper amino acid mixture applied to the skins of guinea-pigs, is absorbed, but only to a very slight extent and with uncertainty, as shown by the presence of small amounts of copper in the liver.

THE RELATION BETWEEN THE ALLERGIC INTRACUTANEOUS REACTION AND THE SYMPTOMS OF ANAPHYLAXIS*

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The intracutaneous reaction of the sensitized animal may be explained thus: The reinjection of the allergen gives rise, at the site of injection, to substances which cause an inflammatory reaction. In this way, the character of the cutaneous reaction is distinguished from the reaction obtained from intravenous reinjection in the guinea-pig, in which the main action is directed against the smooth muscle-fibers; or from that in the dog, in which the lowered blood-pressure is the prominent feature. Altho different species of animals show different symptoms on the reinjection of the allergen, and altho the same animal may show different reactions to the intracutaneous and to intravenous reinjections, it is still conceivable that the same toxin is responsible for the different results. According to this conception, the different results in the different species of animals are explained in this way: In the different species, the toxin seeks out different systems of organs on which to exercise its chief action; for example, the intravenous injections of peptone in dogs and in guinea-pigs give rise to different symptoms. In the case of the same species in which the reinjection is intracutaneous or intravenous, the explanation of the difference in results is that the same toxin gives rise to different lesions in the different organs with which it comes in contact.

On the other hand, a different conception is possible: The reinjection of allergen in different species of animals, or in the same animal, in the case of different modes of injection, leads to the formation of different toxins; the different symptoms in this event being, therefore, natural.

I have attempted to see if it is possible, by means of the intracutaneous reaction, to obtain evidence for one or other of these conceptions. The question on which the experiments were based, was this: Do guinea-pigs and rabbits give constantly a definite reaction on the

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intracutaneous injections of those poisons, or mixtures of poisons, which, on the intravenous injection in the guinea-pig, give rise to general symptoms regarded as characteristic of anaphylaxis? The poisons chosen for the experiments were: B-imidazolyl ethylamin, Witte's peptone, the toxic protein of Vaughan, and the anaphylatoxin of Friedberger.

EXPERIMENTS WITH B-IMIDAZOLYL ETHYLAMIN

The preparation¹ used was histamin hydrochlorid (Hoffmann, LaRoche Co.). Of this preparation 0.5 mg. was sufficient, on intravenous injection, to cause immediate death in a guinea-pig, weighing 500 gm. The intracutaneous injection of 0.1 c.c. of a 0.1 percent neutral solution of this preparation (0.0001 of histamin hydrochlorid) gave negative results in 11 rabbits; as did 0.1 c.c. of a 2 percent solution (0.002 gm.) in 9 rabbits. Four guinea-pigs received 0.1 c.c. of a 1 percent solution intracutaneously without a local reaction; two of these animals died within a few hours. These results confirm those of Müller,² who found that histamin does not cause a reaction on intracutaneous injection.

EXPERIMENTS WITH WITTE'S PEPTONE

Ten percent solutions of different preparations were used. Of these, 3-5 c.c., on intravenous injection, always caused death immediately, or within a few minutes, in guinea-pigs of 200-600 gm.

Pfeiffer and Mita³ obtained marked intracutaneous reactions on the injection of large amounts of Witte's peptone. The results of my experiments, in which a large number of rabbits and guinea-pigs received intracutaneous injections of 1 c.c. of the neutralized solutions, coincide with theirs. The results on the injection of smaller amounts are less definite. In the intracutaneous injection of peptone, as well as of other substances, the reaction of the solution must be considered, as the different preparations vary greatly in this respect.

One of the preparations, which was strongly alkaline, caused marked reactions in 6 rabbits on the injection of 0.1 c.c. A neutralized solution of the same preparation gave no reaction in 2 out of 3 rabbits, and a slight reaction in the third. Rabbits and guinea-pigs, on the injection of 0.1 c.c. showed, in the majority of cases, negative or very faint reactions.

EXPERIMENTS WITH THE "TOXIC PROTEIN" OF VAUGHAN

A. Preparations from egg albumin.—A guinea-pig weighing 205 gm. died within 3 hours after an intraperitoneal injection of 20 mg. of a preparation from egg albumin.

Fifteen rabbits received intracutaneous injections of 0.1 c.c. of a neutralized 4 percent solution. The majority of these showed no reactions, while, in a few, the results were doubtful. None of the fifteen gave a definite reaction.

Of 11 guinea-pigs, which received intracutaneous injections, two gave very slight reactions. The others were negative.

A second preparation was less toxic; of this, however, 50 mg. were sufficient to cause immediate death, when injected intravenously in a guinea-pig, weighing 420 gm.

1. Jour. Physiol., 1910, 40, p. 38; Ibid., 1910, 41, p. 318; Ibid., 1911, 43, p. 182.

2. Ztschr. f. Immunitätsf., 1913, 18, p. 185.

3. Ibid., 1909, 4, p. 410.

4. Jour. Infect. Dis., 1907, 4, p. 476; Ztschr. f. Immunitätsf., 1909, 1, p. 25; Jour. Am. Med. Assn., 1914, 62, p. 583.

Six rabbits were given intracutaneous injections of 1 c.c. of a neutralized 10 percent solution (i. e. 0.1 gm.). Of these, one gave a definite, tho moderate, reaction; the others showed negative, or very slight reactions.

Three guinea-pigs showed negative reactions, after 0.5 c.c. was given intracutaneously; three others showed negative, or indefinite, reactions after 1 c.c.

B. Preparations from tubercle bacilli.—I am indebted to Dr. Vaughan for this preparation. The M. L. D., on intracardial injection in guinea-pigs, was given as 0.00076 gm. A guinea-pig, weighing 340 gm., on the intravenous injection of 2 mg. in a neutralized solution, died before the end of the injection. In the calculation of the amount of the toxic protein injected, the protein not going into the solution was not considered.

Six rabbits received intracutaneous injections of 0.2 c.c. of the 10 percent neutralized solution (0.02 gm.), without showing a definite reaction. Six of the same animals received 0.5 c.c. (0.05 gm.). None gave a definite reaction. Of 7 guinea-pigs, only one gave a moderate reaction on the injection of 0.2 c.c.

EXPERIMENTS WITH FRIEDBERGER'S ANAPHYLATOXIN

A. Anaphylatoxin obtained with edestin (White and Avery).⁵—A guinea-pig, weighing 235 gm., received 3 c.c. intravenously of this preparation; after definite symptoms the animal recovered.

Four rabbits and 2 guinea-pigs received 0.1 c.c. on intracutaneous injection. No reaction was shown.

B. Anaphylatoxin from typhoid bacilli.—Müller² reports his results with the intracutaneous injection of an anaphylatoxin, such as this, in normal and tuberculous guinea-pigs, without, however, giving data as to the general toxicity of the preparation. As he obtained similar slight reactions to the control injections of inactivated serum, he expresses the belief that anaphylatoxin does not possess the power to excite inflammation. With a preparation obtained by the treatment of bacilli with salt solution, he found a somewhat more definite reaction in a tuberculous animal than in a normal one.

Dold and Rados⁶ found that anaphylatoxin, obtained from bacteria, caused inflammation in the eyes of rabbits.

I made an anaphylatoxin by the treatment of dead typhoid bacilli with fresh guinea-pig serum. The intravenous injection, in a guinea-pig of 210 gm., of 2 c.c. of the fluid, obtained after centrifugation, led to slight symptoms of short duration. Six rabbits received 0.1 c.c. intracutaneously. Of these, one gave a definite reaction; two, marked reactions; and three, slight.

Fluid for control experiments was obtained by the treatment of dead bacilli with physiological salt solution. The intracutaneous injection of 0.1 c.c. of this fluid in 7 rabbits gave a marked reaction in 4 animals, a definite one in two, and a slight reaction in one. From this it may be concluded that the reactions, obtained with the anaphylatoxin, cannot be ascribed to its action. The results of the control experiment led to the discontinuation of further work with anaphylatoxin from bacteria.

C. Anaphylatoxin prepared according to the directions of Friedberger and Vallardi.⁷—This anaphylatoxin was obtained by the treatment, with fresh guinea-pig serum, of the precipitate obtained from the interaction of horse serum with the serum of rabbits immunized to horse serum. A number of different preparations were used: 1, 2, 3, 4, and 5.

5. Jour. Infect. Dis., 1913, 13, p. 103.

6. Deutsch. Med. Wchnschr., 1913, 39, p. 1492.

7. Ztschr. f. Immunitätsf., 1910, 7, p. 94.

1. The intravenous injection of 4.5 c.c. of this preparation in a guinea-pig of 210 gm. led to marked symptoms with convulsions lasting one hour; the animal recovered.

Five rabbits received 0.1 c.c. intracutaneously; three of these showed no reaction, two gave reactions. The 2 rabbits giving reactions gave equally marked positive reactions to the control injections of 0.1 c.c. normal guinea-pig serum, which they received at the same time.

Five guinea-pigs received 0.1 c.c. without any reactions.

2. The intravenous injection of 5.0 c.c., in a guinea-pig of 219 gm., led to moderate symptoms, followed by recovery.

Of 6 rabbits, each of which received 0.2 c.c. intracutaneously, five gave no reaction and one a moderate one. The injection, at the same time of 0.2 c.c. normal guinea-pig serum, gave entirely negative results.

3. The intravenous injection of 5.0 c.c. in a guinea-pig of 200 gm. caused immediate death. Six rabbits, on the intracutaneous injection of 0.2 c.c., gave no reaction.

4. The intravenous injection of 4.0 c.c. in a guinea-pig of 180 gm. led to death within a few minutes.

Six rabbits were given intracutaneous injections of 0.2 c.c.; three of these gave reactions, three did not. At the same time, these animals received 1 c.c. of normal guinea-pig serum intracutaneously; this, as in all the experiments, was treated just as was that used to prepare the anaphylatoxin, except that the addition of the precipitate was omitted. The result was that those animals that gave reactions to anaphylatoxin, also gave them to normal guinea-pig serum.

These 6 rabbits also received each 1 c.c. anaphylatoxin intracutaneously; two did not give reactions, four did. Control injections of 1 c.c. normal guinea-pig serum, as before stated, gave positive reactions in 3 of these 4 animals, while in 1 rabbit the control injection was without effect. This was the only case in all the experiments in which an animal giving a decided reaction to anaphylatoxin did not also give a reaction to normal guinea-pig serum.

Six guinea-pigs received 0.1 c.c. without giving any reaction.

5. The intravenous injection of 3.5 c.c. in a guinea-pig of 197 gm. caused immediate death.

Three rabbits received intracutaneous injections of 0.5 c.c.; two gave fairly marked reactions, and one a slight reaction. In one animal the reaction continued to increase after 24 hours, and cultures and smears showed it to be of an infectious character. I wish to note that all the anaphylatoxin and normal guinea-pig serum were obtained and kept under sterile precautions, and were proved sterile by cultures. The control injections of 0.5 c.c. normal guinea-pig serum caused reactions of the same intensity as those of the anaphylatoxin.

In the experiments with Preparations 2, 3, and 4, the same 6 rabbits were used. The experiments were carried out on February 8, 11, and 14. We see that one-half of the rabbits, which gave negative reactions to both anaphylatoxin and normal guinea-pig serum on the first injection, gave positive reactions to both on the third injection, six days later.

For Experiment 3, this is of no importance; for Experiment 4, on February 14, however, it may have to be taken into consideration. Six control animals were treated in exactly the same way with injections of guinea-pig serum. One of these animals died. Of the remaining five, one, on the fifth day, showed a marked reaction, and two, moderate reactions to 1.0 c.c. of serum, while 0.2 c.c. was without effect. Four fresh rabbits were then given intracutaneous injections of 0.1 c.c. horse serum every second day. Of these, one showed a marked

reaction as early as the third injection (on the fifth day); one at the fifth injection (on the eighth day); while the other two began to show slight reactions at the sixth injection. For Experiments 1 and 5, fresh rabbits were used.

Friedberger⁸ believes that the subcutaneous injection, even of small amounts of anaphylatoxin, easily leads to the Arthus phenomenon; he does not, however, report controls with normal guinea-pig serum. My experiments, as far as they go, hardly speak in favor of any action of anaphylatoxin causing inflammation.

The results of the experiments may be summarized thus: Primarily toxic substances, which on intravenous injection in guinea-pigs give rise to symptoms identical with, or similar to, those of anaphylaxis, given intracutaneously in guinea-pigs and rabbits, do not necessarily cause inflammation; while the sensitized animal (guinea-pig, rabbit, or dog) under certain conditions, reacts, with an inflammation, to an intracutaneous injection of the allergen. The substances giving rise to the inflammation owe their origin to an inter-reaction between the organism and the allergen.

According to the theory of Vaughan, and later of Biedl and Kraus,⁹ Friedberger,⁸ Pfeiffer,¹⁰ Schittenhelm and Weichardt,¹¹ and others, the production of the anaphylactic poisons depends on a splitting of protein; the substances used by me also derive their origin primarily from a cleavage of protein. In this respect, they are differentiated from a number of other substances, which, for example, an intravenous injection in guinea-pigs, may give rise to a distension of the lung. The possibility that they are entirely unlike the poison formed in anaphylaxis is, however, not absolutely excluded, as has been claimed by Friedberger.⁸ In proving the identity of a poison with those formed in anaphylaxis, it is often required that it should show a whole series of actions, using the symptoms observed in anaphylaxis as a criterion. This is going too far. Schittenhelm and Weichardt remark that in the process of protein splitting, during the course of which we reckon upon the appearance of the anaphylactic poisons, we must expect the formation of a number of other substances which contribute to the whole picture of anaphylaxis, while they need not participate in the principal toxic action. As examples of such concomitant features in the complex picture of anaphylaxis, there may be mentioned the

8. *Ibid.*, 1913, 18, p. 227.

9. *Ibid.*, 1911, 10, p. 711.

10. *Ibid.*, 1913, 16, p. 38; *Ibid.*, 1911, 10, p. 550; *Ztschr. f. d. ges. Neurol. u. Psychiat.*, 1912, 9, p. 409.

11. *München. med. Wchnschr.*, 1912, 59, pp. 67, 1089; *Ztschr. f. Immunitätsf.*, 1912, 14, p. 609.

increase in coagulation time of the blood and the eosinophilia, described by Schlecht,¹² which occurs in sensitized guinea-pigs, which do not die on reinjection. This idea is the more plausible, in that antianaphylactic animals also react to repeated reinjections with a marked eosinophilia. This may be ascribed to the action of the by-products of protein splitting and not to that of the principal poisons. Therefore, we must agree with Schittenhelm and Weichardt, Ahl and Schittenhelm,¹³ Vaughan, and others, that the absence of one or other of the symptoms does not necessarily speak against the identity of certain toxic substances with the poisons formed in anaphylaxis. Of course, the proof of identity rests finally on the chemical analysis.

If, however, we adopt the point of view that the substances with which I have worked, possess a close resemblance to those poisons formed on the introduction of the allergen in the circulating blood in anaphylaxis, then we are led to conclude that, in the intracutaneous reaction, the formation of substances causing inflammation comes into the foreground, and that these substances are different from those formed, for example, in the guinea-pig on intravenous injection of the allergen, and showing their action chiefly on the smooth muscles.

From the experiments of Dale,¹⁴ who worked with the isolated lung of guinea-pigs, we may conclude that the muscle takes part in the production of the poison. The results of Manwaring¹⁵ and still more, those of Voegtlin and Bernheim,¹⁶ confirmed by those of Denecke,¹⁷ also indicate that the tissues participate in the formation of the anaphylactic poisons. According to these authors, the formation of the substance causing a fall in blood-pressures in the dog does not occur without the action of the liver. The so-called cellular theory of toxin formation finds further evidence in the work of Coca,¹⁸ and of v. Fenyvessy and Freund.¹⁹ My results, also, under the supposition mentioned, seem to indicate that the tissues, in which an inflammation occurs on local injection, take part in the formation of the poisons; and that it is the difference in the tissues taking part in the formation of the poisons that causes the difference in poisons. As far as we may conclude at present, the skin does not behave differently from some

12. Arch. f. exper. Path. and Pharmacol., 1911, 67, p. 137.

13. Ztschr. f. ges. exper. Med., 1913, 1, p. 111.

14. Jour. Pharmacol. and Exper. Therap., 1912, 4, p. 167.

15. Ztschr. f. Immunitätsf., 1911, 8, p. 1.

16. Jour. Pharmacol. and Exper. Therap., 1911, 2, p. 507.

17. Ztschr. f. Immunitätsf., 1914, 20, p. 501.

18. Ibid., 20, p. 662.

19. Ibid., 22, p. 59.

other organs; for example, the lung of guinea-pigs, which, on local administration of the allergen, shows an inflammatory reaction.²⁰

However, the identity of the substances causing inflammation in the different tissues, as, for example, in skin and lungs, is not proved. It is not impossible that the skin of the sensitized animal shows certain peculiarities. I attempted to obtain some evidence on this question through the study of the course of the intracutaneous reaction in antianaphylactic guinea-pigs. It is known that large doses of horse serum, given intravenously, depress the intracutaneous reaction of the sensitized rabbit.²¹ It is to be noted, in this connection, that I found horse serum to have no influence on the intracutaneous inflammation caused by mustard oil. After a few days, however, the reactivity of the skin returns. It was my intention to determine whether the intracutaneous reaction in the guinea-pig would reappear again at a time when the animals were still resistant to the intravenous injection. As two large series of experiments, with animals of about equal weights, proved unsatisfactory on account of the extreme differences in degree of sensitization, I was obliged to give up this experiment. I found, however, a number of sensitized animals, which gave no intracutaneous reactions, altho they suffered fatal anaphylactic shock on intravenous reinjection of the allergen. Dr. Amberg tells me that similar conditions have been seen in several dogs that reacted promptly, with a marked fall in blood-pressure, to an intravenous reinjection of the allergen.

In this case, other tissue elements than the muscles seem to be involved chiefly in the formation of the poison — and this is what concerns us here.

The fact that Witte's peptone must be included among the substances causing inflammation seems to offer some difficulties to the conception. But as in the supposed splitting of protein, which underlies the formation of these poisons, reactions occur giving the freest play to all possible variations, we should not be surprised to find among the sum of split products, obtained under different conditions, a combination which contains not only the generally toxic components, but also those causing local inflammation. On the other hand, histamin and the protein poison of Vaughan, whose mother substances, at least, are split products of protein, seem to lack the power to cause inflammation. My preparations of anaphylatoxin seem also to resemble the latter substances. Still, on account of quantitative relations, I do not wish, at present, to attach too much importance to these experiments. The conception that different tissues may give rise to different split products from the same zymot presupposes the presence of different enzymatic actions. That this is no impossible condition is shown, for

20. Friedberger, *Ztschr. f. Immunitätsf.*, 1910, 7, p. 94; *Ibid.*, 1913, 18, p. 227; Ichioka, *Deutsch. Arch. f. klin. Med.*, 1912, 107, p. 500; Busson, *Wien. klin. Wchnschr.*, 1911, 24, p. 1492; Schlecht and Schwenker, *Deutsch. Arch. f. klin. Med.*, 1912, 108, p. 405; and Ströbel, *München. med. Wchnschr.*, 1912, 59, p. 1538.

21. Amberg and Knox, *Jour. Pharmacol. and Exper. Therap.*, 1912, 3, p. 223.

example, by the splitting of nucleic acid by different organs. In this case, the substance in question possesses a relatively simple constitution as compared with that of the proteins. In the normal protein metabolism in the organism we must also reckon upon a large number of enzymes; still others, as has been shown by Abderhalden and others, appear under abnormal circumstances, conditions such as prevail in the sensitized animal. It is not impossible that the split products, which we consider necessary for the anaphylactic reactions, do not arise from the allergen at all; but that the tissue protein may take part in their formation. Under such circumstances, the appearance of poisons, differing according to the tissues having part in their formation, is still more easily understood.

CONCLUSIONS

On the ground of the conception that the anaphylactic reactions are caused by substances in close relation to the parenteral splitting of protein, the experiments, detailed in this paper, speak in favor of the hypothesis that the intracutaneous reaction of the sensitized animal is *not* caused by the same split-products which lead to the general symptoms of anaphylaxis on the reinjection of the allergen into the bloodstream.

THE PROTEIN POISON OF THE TONSIL *

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In 1913, Dick and I found that the extracts of tonsils were, in many instances, acutely toxic for animals when injected intravenously.¹ This toxic action manifested itself clinically like the reaction obtained in anaphylaxis. Bacteriological examinations showed that typical hemolytic streptococcus colonies could be obtained in the flora of the highly toxic tonsils whereas this organism was present in only 10 percent of the relatively non-toxic tonsils. It was noted, among other things, that, unlike the anaphylatoxin of Friedberger, the toxicity of these extracts was not destroyed by heating to 65 C., but that it was diminished without rendering them entirely non-toxic. A further consideration of this point has led me to believe that the toxicity of these extracts is dependent upon more than one factor. That the toxicity of these extracts is dependent, in part, upon an anaphylatoxin of bacterial extraction is not improbable. The nature of the heat resistant portion, however, is open to question.

With this in view a further study of tonsil extracts was undertaken. The tonsils were obtained through the courtesies of Dr. A. A. Hayden and Dr. Lillian E. Taylor. From a study of Table 1, it will again be seen that, in a large majority of the extracts of greatest toxicity, the streptococcus hemolyticus was present, and that it was usually absent or, at most, not the prevailing organism, in the tonsils from which the less toxic extracts were obtained.

From a gross examination of the tonsils, it was noted that tonsils with well-defined crypts, and especially those containing caseous plugs, were almost uniformly severely toxic. It was also observed, in most instances, that these extracts contained a greater amount of heat-non-coagulable-biuret reacting substances than did the less toxic tonsils. Without exception, all organ and tissue extracts contain such substances, but the marked increase in the depth of the biuret reaction was most striking when compared with that of the less toxic extracts.

* Received for publication September 1, 1914.
1. Jour. Infect. Dis., 1913; 13, p. 273.

TABLE 1
APPEARANCE AND FLORA OF TONSILS, AND ACTION OF EXTRACT

Tonsil	Gross Appearance of Tonsil	Bacterial Flora of Tonsil	Biuret Reaction of Tonsil Extract	Amount Injected	Action on Rabbit
1	Large, hyperplastic, no caseous plugs	Staphylococcus, micrococcus tetragenus, influenza bacillus	+	5 c.c.	Sneezed, urinated, rapid respiration, quiet, lived 2 minutes
2	Large, hyperplastic, crypts, caseous plugs	Hemolytic streptococcus, small punctate colonies, staphylococcus, streptococcus mucosus	+++	4 c.c.	Died 2 minutes
3	Large, hyperplastic, no caseous plugs	Staphylococcus, pneumococcus, hemolytic streptococcus, small punctate colonies, very few	+	6 c.c.	Sneezed, urinated, died during night
4	Large, hyperplastic, no plugs	Staphylococcus, streptococcus viridans, B. Friedlander, hemolytic streptococcus, small punctate colonies	+	10 c.c.	Lived, restless, rapid breathing
5	Large, hyperplastic, no plugs	Staphylococcus, streptococcus viridans, hemolytic streptococcus, small punctate colonies	++	9 c.c.	Lived, restless, rapid breathing, defecated, urinated
6	Small, fibrous, no plugs	Hemolytic streptococcus, small punctate colonies, streptococcus viridans, staphylococcus, pneumococcus	+++	5 c.c.	Died, 1 minute
7	Large, hyperplastic, scars, no plugs	Staphylococcus, hemolytic streptococcus, small punctate colonies	+++	5 c.c.	Died, 30 seconds
8	Large, hyperplastic, no plugs	Streptococcus viridans, staphylococcus, pseudodiphtheria bacilli, pneumococcus	++	10 c.c.	Lived, slight twitching
9	Large, hyperplastic, caseous plugs, scars	Hemolytic streptococcus, small punctate colonies, streptococcus viridans, staphylococcus, pneumococcus	+++	5 c.c.	Died, 30 seconds
10	Large, hyperplastic, no plugs	Hemolytic streptococcus, large, low, flat, wrinkled colony, staphylococcus	++	9 c.c.	Lived, slight twitching, scratched nose and ears
11	Large hyperplastic, caseous plugs	Hemolytic streptococcus, small punctate colonies, staphylococcus, pseudodiphtheria bacilli	+++	4 c.c.	Died, 1 minute
12	Large, hyperplastic, caseous plugs	Staphylococcus, hemolytic streptococcus, small punctate colonies	++	10 c.c.	Died, 8 minutes
13	Small, fibrous	Staphylococcus, streptococcus viridans, micrococcus catarrhalis	+	5 c.c.	Lived, quiet, urinated, defecated
14	Large, hyperplastic, caseous plugs	Hemolytic streptococcus, large, low, flat, wrinkled colony, hemolytic streptococcus, small, punctate colonies, staphylococcus alba	+++	5 c.c.	Died, 30 seconds
15	Small, fibrous, scars	Staphylococcus, pneumococcus, hemolytic streptococcus, small punctate colonies	++	5 c.c.	Died, 3 minutes
16	Large, hyperplastic, no plugs	Hemolytic streptococcus, small punctate colonies, staphylococcus, pneumococcus	+++	5 c.c.	Died, 7 minutes
17	Small, fibrous, no crypts	Influenza bacillus, micrococcus tetragenus, pneumococcus, staphylococcus	+++	5 c.c.	Died, 1 minute
18	Large, hyperplastic, crypts, caseous plugs	Hemolytic streptococcus, large, low, flat, wrinkled colony, hemolytic streptococcus, small punctate colonies, staphylococcus alba	++++	5 c.c.	Died, 1 minute
19	Small, hyperplastic, crypts, caseous plugs	Pneumococcus, non-hemolytic streptococcus, small punctate colonies, staphylococcus	+++	5 c.c.	Died, 2 minutes

TABLE 1—Continued

20	Large, hyperplastic, crypts, caseous plugs	Pneumococcus, hemolytic streptococcus, small punctate colonies, streptococcus viridans, staphylococcus	++++	5 c.c.	Died, 1 minute
21	Large, fibrous, no plugs	Staphylococcus, pneumococcus, hemolytic streptococcus, large, low, flat, wrinkled colony	++	5 c.c.	Lived, rubbed nose, face and ears, urinated, defecated, rapid respiration
22	Very large, hyperplastic, caseous plugs	Hemolytic streptococcus, large, low, flat, wrinkled colony, staphylococcus	++++	5 c.c.	Died, 15 seconds
23	Large, hyperplastic, crypts, no plugs	Streptococcus viridans, staphylococcus, hemolytic streptococcus, small punctate colonies, few scattered	++	5 c.c.	Lived, scratched nose and ears, urinated, defecated, slight twitching
24	Large, hyperplastic, no caseous plugs	Hemolytic streptococcus, large, low, flat, wrinkled colony, staphylococcus aureus	+++	5 c.c.	Lived, rubbed nose, sneezed, urinated, panting respiration
25	Large, hyperplastic, caseous plugs	Hemolytic streptococcus, large, low, flat, wrinkled colony, B. Friedländer, staphylococcus	++++	5 c.c.	Died, 30 seconds
26	Large, hyperplastic, no caseous plugs	Staphylococcus, streptococcus viridans	+++	5 c.c.	Lived, sneezed, rubbed ears, face and nose, urinated, defecated, panting respiration
27	Large, hyperplastic, caseous plugs	Pneumococcus, staphylococcus	+	5 c.c.	Lived, urinated, no other symptoms
28	Large, hyperplastic, crypts, no plugs	Staphylococcus, hemolytic streptococcus, large, low, flat, wrinkled colony, incomplete hemolysis	++	5 c.c.	Lived, panting respiration, no other symptoms
29	Large, hyperplastic, no caseous plugs	Staphylococcus, non-hemolytic streptococcus, small punctate colonies, pseudodiphtheria bacilli	+	5 c.c.	Lived, shallow rapid respiration, no other symptoms
30	Large, hyperplastic, caseous plugs	Streptococcus viridans, pneumococcus, staphylococcus	++++	5 c.c.	Lived, shallow rapid respiration, no other symptoms
31	Large, hyperplastic, caseous plugs	Staphylococcus, streptococcus viridans	++	5 c.c.	Lived, sneezed, urinated, defecated, rubbed nose and face, shallow panting respiration
32	Small, fibrous, no caseous plugs	Staphylococcus, streptococcus viridans	+	5 c.c.	Lived, sneezed, defecated, panting respiration, rubbed nose
33	Medium sized, no plugs	Staphylococcus, pneumococcus, hemolytic streptococcus, small punctate colonies	+++	5 c.c.	Died, 3 minutes
34	Hyperplastic, caseous plugs	Hemolytic streptococcus, small punctate colonies, staphylococcus	+++	5 c.c.	Died, 1 minute
35	Very large, hyperplastic, no plugs	Streptococcus viridans, staphylococcus, hemolytic streptococcus, small punctate colonies	++	5 c.c.	Died, 1 minute
36	Large, hyperplastic, caseous plugs	Hemolytic streptococcus, small punctate colonies, staphylococcus	+++	5 c.c.	Died, 1 minute
37	Medium sized, no plugs	Staphylococcus, streptococcus viridans, pneumococcus	+	5 c.c.	Lived, sneezed, urinated, dyspnea
38	Medium sized, no plugs	Staphylococcus, B. Friedländer, non-hemolytic streptococcus, large, low, flat, wrinkled colony	++	5 c.c.	Lived, rubbed nose, sneezed, urinated, defecated, panting respiration
39	Small, fibrous, no plugs	Staphylococcus, micrococcus catarrhalis	+	5 c.c.	Lived, urinated, sneezed
40	Large, hyperplastic, caseous plugs	Staphylococcus, hemolytic streptococcus, small punctate colonies	++++	5 c.c.	Died, 15 seconds

Those organisms which were present in greatest numbers are listed in the order of their frequency. The number of + signs express the relative degree of positive reaction of the individual extracts.

In determining this reaction the extracts were treated according to the method of Pfeiffer and Mita.² Less than 4 c.c. of the extract were diluted to ten times its volume with water and placed in an Erlenmeyer flask. This was immersed in boiling water and a very dilute acetic acid was added, drop by drop, until the mixture just reacted acid to litmus. It was found that before this point was reached the mixture invariably separated into coarse coagula and a water clear fluid, which could be filtered very readily. This was, by far, the more important consideration, and when the coagulation and separation were found to have taken place the addition of acid was stopped. The filtrate was then heated 15-30 minutes longer in the water bath, and tested with nitric acid for albumen. In all instances, the ring test was negative. Twenty cubic centimeters of the filtrate were then evaporated to 2 c.c. and tested with biuret reagent.

With few exceptions, the stronger biuret reactions were obtained with the more toxic extracts, altho biuret reactions of varying degrees and intensities were obtained in all extracts, as shown by Table 1. In other words, the more toxic extracts contained a larger amount of peptone-like products.

Biedl and Krauss,³ in trying to determine the nature of anaphylatoxin, found that, by injecting peptones into animals, a condition of shock was produced which resembled anaphylaxis. Vaughan and Wheeler⁴ were able to obtain, from the colon bacillus and other pathogenic and non-pathogenic bacteria, a protein-split product of marked toxicity which, when injected intravenously in doses of 0.5 mg., killed guinea-pigs under conditions resembling anaphylaxis clinically. They called this toxic substance "the protein poison" and advanced the theory that symptoms, due in general to bacterial disease, could be explained on the basis of a parenteral splitting of the bacterial proteins into toxic substances, akin to peptones. This theory has been accepted by many workers in the field of infectious diseases. Provided the individual has become sensitized, that is, has developed the specific lytic bodies for a certain protein, symptoms of protein poison can be produced in such an individual by the parenteral ingestion of the same protein. This is possible not only for bacterial proteins, but for proteins in general. It has been possible to sensitize animals and to produce the symptoms of protein poisoning with proteins derived from their own tissues. Schittenhelm and Stroebel⁵ found that the action of pepsin on species-similar tissue resulted in the formation of toxic-split products. Heyde and Vogt,⁶ by a series of very interesting experiments, have shown

2. *Ztschr. f. Immunitätsf.*, 1910, 6, p. 18.

3. *Wien. klin. Wchnschr.*, 1909, 10, p. 363.

4. *Jour. Am. Med. Assn.*, 1913, 61, p. 1761.

5. *Ztschr. f. exper. Path. u. Therap.*, 1912, 11, p. 108.

6. *Ztschr. f. d. ges. exper. Med.*, 1913, 1, p. 59.

that death, in cases of burns, resulted from aseptic tissue splitting, and the production of a condition resembling anaphylactic shock as a result of the absorption of the toxic products. They also found that by crushing the animal's own tissue it was possible to sensitize the individual to this tissue.

By applying Abderhalden's dialyzing and optical methods for the detection of protein splitting by serum in different conditions, a great volume of experimental work has been produced, indicating that specific homologous proteolysis does occur and may be an important factor in the clinical manifestations of disease. Rollman⁷ advances the theory that disease of one of a pair of organs, especially in suppurations, results in the production of specific proteolytic ferments, which are capable of producing destructive changes in the other organ. It would seem not unlikely that the parenteral absorption of tissue proteins and their parenteral splitting, or the absorption of peptones, products of their splitting in loco, must be of some moment. This does not apply only to paired organs, but is equally applicable to a continued breaking down of tissue in any part of the body.

With this in mind, it was determined to ascertain the splitting power of the sera of individuals, the subjects of chronic and recurrent tonsillitis, on their own tonsil tissue, as well as on other tissue; and to determine, if possible, whether or not there is any proportional relationship between this splitting power, the toxicity of the tonsillar extracts, and their content of peptone-like bodies.

The patients were bled just previous to the tonsillectomy. The tonsils, after removal, were washed as free from blood as possible in a running stream of water. Each pair of tonsils was then rubbed up in a sterile mortar with 20 c.c. of physiological salt solution under aseptic conditions. The extracts were then decanted, vigorously centrifuged at 3,000 revolutions per minute for 10-15 minutes, and again decanted. Both residues were then boiled in water, according to Abderhalden's method of preparing placental tissue,⁸ until they were free from water-soluble ninhydrin and biuret reacting substances. About 1 gm. of the prepared tonsil tissue was then placed in a dialyzing sac, and covered with 1.5 c.c. of the serum. The sac and its contents were then placed in a receptacle containing 20 c.c. of distilled water; both water and contents of the sac were covered with a layer of toluene, and placed in an incubator at 37 C. for 16-18 hours. The dialyzing sacs were prepared from a thin 6-7 percent solution of celloidin in a mixture of equal parts of alcohol and ether. Only such celloidin was used as would permit a dialysis from a solution of silk peptone and would retain albumens, as evidenced by a failure of dialysis of biuret

7. Deutsch. med. Wehnschr., 1913, 39, p. 2239.

8. Handb. d. Biochem. Arbeitsmethoden, 1912, 6, p. 226.

TABLE 2
 PROTEOLYTIC POWER OF SERUM AS COMPARED WITH ACTION TONSILLAR EXTRACT

Tonsil	Tonsillar Extract		Splitting Power of Active Serum on Homologous Tonsil	Splitting Power of Active Serum on Heterologous Tissue	Splitting Power of Rabbit Serum on Tonsil and Other Tissue
	Amount	Action on Rabbit			
1	5 c.c.	Slight, lived	B+ N+
2	4 c.c.	Killed	B+ N+++
4	10 c.c.	Lived	B- N-
5	9 c.c.	Lived	B+++ N+++	Tonsil 7, B+++
6	5 c.c.	Killed	B+++ N+++	Tonsil 7, B++++, N++++
7	5 c.c.	Killed	B- N+	Tonsil 5, B-, N+
8	10 c.c.	Lived	B+ N+++	Tonsil 11, B+++
10	9 c.c.	Lived	B- N+	Kidney B-, N+ Lymph-gland B-, N-
11	4 c.c.	Killed	B- N+	Lymph-gland B-, N+ Kidney B-, N+++ Tonsil 8, B-, N+
16	5 c.c.	Killed	B- N-	Spleen B-, N- Thymus B-, N- Kidney B-, N- Ovary B-, N-	Tonsil 16 B-, N- Kidney B-, N-
17	5 c.c.	Killed	B+++ N+++	Thymus B+, N++	Tonsil 17 B++++, N++++ Thymus B-, N-
18	5 c.c.	Killed	B- N+	Thymus B-, N-	Tonsil 18 B-, N-
19	5 c.c.	Killed	B+++ N+++	Thymus B+, N+++ Spleen B-, N+++ Kidney B+, N+++ Ovary B-, N+	Tonsil 19 B-, N+ Thymus B-, N+++
20	5 c.c.	Killed	B- N+	Thymus B+, N+++ Spleen B-, N+++ Kidney B-, N+	Tonsil 20 B-, N+++ Spleen B-, N- Kidney B-, N-
21	5 c.c.	Lived	B- N-	Tonsil 21 B-, N-

TABLE 2—Continued

22	5 c.c.	Killed	B+++ N+++	Tonsil 22 B—, N—
23	5 c.c.	Lived	B— N—	Tonsil 23 B—, N—
24	5 c.c.	Lived	B— N—	Tonsil 24 B—, N—
25	5 c.c.	Killed	B— N—	Tonsil 25 B—, N—
26	5 c.c.	Lived	B+++ N+++	Kidney B—, N—	Tonsil 26 B—, N+++
27	5 c.c.	Lived	B— N—	Kidney B—, N—	Tonsil 27 B—, N+++
28	5 c.c.	Lived	B— N—	Kidney B—, N—	Tonsil 28 B—, N+++
29	5 c.c.	Lived	B— N—	Kidney B—, N—	Tonsil 29 B—, N—
30	5 c.c.	Lived	B— N—	Kidney B—, N—	Tonsil 30 B—, N+
31	5 c.c.	Lived	B+++ N+++	Kidney B—, N—
32	5 c.c.	Lived	B— N+++	Liver B—, N+++
33	5 c.c.	Killed	B— N+++	Liver B—, N—
34	5 c.c.	Killed	B+++ N+++	Liver B—, N+++
35	5 c.c.	Killed	B— N—	Liver B—, N—
36	5 c.c.	Killed	B— N—	Liver B—, N—
37	5 c.c.	Lived	B+++ N+++	Liver B—, N—
38	5 c.c.	Lived	B— N—	Liver B—, N—
39	5 c.c.	Lived	B— N—	Liver B—, N—
40	5 c.c.	Killed	B— N+	Liver B—, N—

+- = doubtful; — = negative.
 B = biuret reaction of dialysate; several + signs = relative degree of reaction.
 N = ninhydrin reaction of dialysate.

and ninhydrin reacting substances from normal blood serum.⁹ In compliance with the recommendation of Abderhalden¹⁰ not over 1.5 c.c. of serum were used in any of the experiments. The results have been recorded in Tables 2, 3, and 4.

From a study of Table 2 it will be seen that 12 of the 16 most toxic tonsils, the extracts of which killed acutely, were split by the serum obtained from the donors of the tonsils; and that, in all instances, these were tonsils the extracts of which had a large peptone content. From a comparison of Tables 1 and 2 it will also be seen that, in most instances, these were cases in which the tonsils presented well-marked crypts with caseous plugs. Table 2 also shows that, in the 18 less toxic tonsils, the serum-splitting action on tonsil tissue was absent in 9 cases and present in 9 cases; if further comparison of Tables 1 and 2 is made, it is seen that the extracts of the latter were probably of greater toxicity than those of the former, as evidenced by the degree of reaction obtained in the animal experiments, altho these extracts were not sufficiently toxic, in any instance, to produce death acutely with the amounts injected. It will be noted, however, that the content of heat-non-coagulable biuret-reacting substances was no greater in either of these two groups, demonstrating that the peptone content of these extracts cannot be the only factor upon which the toxicity of tonsils depends.

Suitable control experiments were made, and from the record of these in Table 2 it is seen that the more active sera split with equal ease tonsils other than those of the serum donor. It will also be noted that, in some instances, the control tests with other than tonsillar tissue presented positive, tho less marked reactions. Therefore, it appears that either this splitting action is not strictly specific, or else that more than one specific ferment may have been contained in the sera. Thus, it was found that controls of thymus tissue, spleen, ovary, lymph gland, and kidney were occasionally split by the same serum, tho in practically no instance to so marked a degree.

Another point which necessitated consideration was the possibility that the toxicity of these extracts was due to a parenteral splitting action of the proteins of the tonsil extracts by the rabbit serum after the injection into the rabbit. To test this possibility, the rabbits were bled by cardiac puncture to the extent of 6-7 c.c. of blood, on the day previous to their injection. They were given 15 c.c. of physiological salt solution, hypodermatically, following their bleeding, and furnished

9. *Ibid.*, p. 230.

10. *München. med. Wehnschr.*, 1914, 61, p. 401.

Tonsil	Action of Active Serum on Homologous Tonsil	Action of Inactive Serum on Homologous Tonsil	Action of Inactive Serum Plus Complement on Homologous Tonsil	Action of Complement on Same Tonsil and on Other Tissue	Action of Inactive Serum on Heterologous Tissue	Action of Inactive Serum Plus Complement on Heterologous Tissue	Tonsillar Extract Injected	
							Amount	Action on Rabbit
21	B— N—	B— N—	B— N—	Tonsil B—, N— Lymph gland B—, N—	Lymph gland B—, N—	Lymph gland B—, N—	5 c.c.	Lived
22	B+++ N++++	B— N—	B+—? N+++	Tonsil B—, N— Lymph gland B—, N—	Lymph gland B—, N—	Lymph gland B—, N—	5 c.c.	Died
23	B— N—	B— N—	B— N—	Tonsil B—, N— Lymph gland B—, N—	Lymph gland B—, N—	Lymph gland B—, N—	5 c.c.	Lived
24	B— N—	B— N—	B— N—	Tonsil B—, N— Lymph gland B—, N—	Lymph gland B—, N—	Lymph gland B—, N—	5 c.c.	Lived
25	B— N—	B— N—	B— N—	Tonsil B—, N— Lymph gland B—, N—	Lymph gland B—, N—	Lymph gland B—, N—	5 c.c.	Died
26	B+++ N++++	B— N—	B+ N+	Tonsil B—, N— Kidney B—, N—	Kidney B—, N—	Kidney B—, N—	5 c.c.	Lived
27	B— N—	B— N—	B— N—	Tonsil B—, N— Kidney B—, N—	Kidney B—, N—	Kidney B—, N—	5 c.c.	Lived
28	B— N—	B— N—	B— N—	Tonsil B—, N— Kidney B—, N—	Kidney B—, N—	Kidney B—, N—	5 c.c.	Lived
29	B— N—	B— N—	B— N—	Tonsil B—, N—	5 c.c.	Lived
30	B— N—	B— N—	B— N—	Tonsil B—, N— Kidney B—, N—	Kidney B—, N—	Kidney B—, N—	5 c.c.	Lived
31	B+++ N++++	B— N—	B— N+	Tonsil B—, N— Kidney B—, N—	Kidney B—, N—	Kidney B—, N—	5 c.c.	Lived
32	B— N++	B— N—	B— N++	Tonsil B—, N— Liver B—, N—	5 c.c.	Lived
33	B—+? N++++	B— N—	B+ N++	Tonsil B—, N— Liver B—, N—	5 c.c.	Died
34	B+++ N++++	B— N—	B+ N+++	Tonsil B—, N—	5 c.c.	Died
35	B— N—	B— N—	B— N—	Tonsil B—, N—	5 c.c.	Died
36	B— N—	B— N—	B— N—	Tonsil B—, N—	5 c.c.	Died
37	B— N++	B— N—	B— N+	Tonsil B—, N—	5 c.c.	Lived
38	B+++ N++++	B— N—	B— N+	Tonsil B—, N—	5 c.c.	Lived
39	B— N—	B— N—	B— N—	Tonsil B—, N—	5 c.c.	Lived
40	B— N++	B— N—	B— N+	Tonsil B—, N—	5 c.c.	Died

with an abundance of food and water. They appeared perfectly normal on the following day. The serum, after being separated from its coagulum under aseptic precautions, was placed in a refrigerator until the following day and its splitting power determined for the tonsils, the extracts of which were injected. In 3 of the 7 rabbits, which were acutely killed, there was a splitting action in vitro of the tonsil tissue by the rabbit serum. In one of these three, where thymus tissue was used as a control, it was also found that the thymus tissue was split by the rabbit serum. I mention this because it was noted, in addition to the other results typical of anaphylaxis in rabbits, that in practically all acute deaths of rabbits in these experiments small hemorrhages were found in the thymus. The sera of 4 of 8 rabbits, which did not die, split in vitro the tonsil tissue with which they were subsequently injected. From Tables 1 and 2 it is seen that the symptoms manifested by these rabbits, whose sera had a splitting action, were, in a degree, somewhat more marked than those of the remaining four, whose sera failed to split the tonsil tissue.

That the tonsil substrates used must have contained also bacterial proteins of the tonsillar flora is of course true. It, therefore, seemed plausible that some of the evidences of protein-splitting in these substrates might, in part at least, have been due to the action of the serum on the contained bacterial proteins. With this in mind, control experiments of 9 cases were made as follows: 5 drops of tonsil extract, which had not been centrifuged, were smeared evenly over the surfaces of each of 3 blood-agar plates. These were incubated 18 hours and the surface growth washed off with salt solution. The bacteria were then prepared and used as a substrate for their respective tonsils. In these experiments, the amount of bacterial protein must have been many times greater than the amount that may have remained in the tonsil substrates. As seen in Table 4, in only two instances could any evidence of splitting be obtained and in no one instance did this tend to approach the amount of splitting obtained from the tonsil substrates.

It seemed advisable to determine further, if possible, the nature of this splitting action of serum from patients, the subjects of chronic tonsillitis on tonsil tissue. Dick,¹¹ in a study of the proteolytic substances in the blood, found that this proteolysis was almost entirely inhibited by heating the serum at 56 C. for one-half hour; and that it returned in part when complement was added to the heated serum.

I have found that, when serum from patients is heated at 56 C. for one-half hour, the splitting-action on tonsillar tissue is completely inhibited. If, however, the serum is "activated" by the addition of guinea-pig complement, tonsillar tissue is again split, as will be seen from Table 3. Guinea-pig complement, alone, was, in no instance, capable of splitting tonsil tissue. Table 3 shows that the splitting power of the heated serum plus complement failed to equal in intensity the power of the active serum. In all cases in which the active serum failed to split tonsil tissue, the action of inactive serum plus complement, as was to be expected, was also negative.

TABLE 4
ACTION OF SERUM ON TONSIL AND ON TONSILLAR BACTERIA

Tonsil	Splitting Action of Serum on Homologous Tonsil	Splitting Action of Serum on Bacteria from Homologous Tonsil	Tonsil	Splitting Action of Serum on Homologous Tonsil	Splitting Action of Serum on Bacteria from Homologous Tonsil
32	B— N++	E— N—	37	E— N++	B— N—
33	B—+? N++++	B— N+	38	B+ N++++	B— N+
34	B++ N+++++	B— N—	39	B— N—	B— N—
35	B— N—	B— N—	40	B— N++	B— N—
36	B— N—	B— N—

In conclusion, then, it may be said that the hemolytic streptococcus is present in tonsils, the extracts of which are most toxic, and it is logical that the extracts' toxicity must depend, in part, on toxic products elaborated out of the streptococcus itself, or by the action of the streptococcus out of the tissue in which it is growing; or, that both hypotheses may be true. A further study of this phase should be undertaken.

The tonsils, the extracts of which are most toxic, contain a considerable amount of heat-non-coagulable biuret reacting substance or substances, in all probability a product of the splitting of proteins in loco. This splitting, no doubt, is due in part to the action of saprophytic or pathogenic micro-organisms on the tissue of the tonsil. It is not improbable, however, that part of this splitting action may be due also to the action of a ferment for tonsillar tissue contained in the blood, and which resembles an amboceptor and requires complement to

complete its action. It is plausible to assume that local death of tonsillar tissue by the action of organisms, like the streptococcus, permits of the parenteral absorption of tonsil protein, and results in the formation of an amboceptor for this protein which, with the aid of the complement, is capable of splitting the protein. This splitting action, then, might occur either in loco, or, following the parenteral absorption of tonsil protein, in the circulating blood. The toxic action of tonsillar products may then, in part at least, be responsible for many of the clinical symptoms manifested in the course of disease of the tonsils. The individual becoming sensitized to his tonsils exhibits, from time to time, mild or severe symptoms due to the toxic products of tonsillar origin.

The effect on the individual of continued sensitization in this manner can only be speculated on. Longcope has been able to produce an interstitial hepatitis somewhat resembling a cirrhosis,¹² a myocarditis with scar formation and a glomerular and a parenchymatous nephritis¹³ in rabbits, cats, guinea-pigs, and dogs by repeated sensitization with proteins. The frequency with which myocardial and renal lesions are found accompanying and following acute and chronic tonsillar conditions has long been known. These secondary conditions have been attributed usually to a systemic bacterial invasion with the tonsil as the atrium. This, no doubt, is, in most cases, the predominant etiological factor. It does not seem improbable, however, that some of these conditions may be due, in part at least, to toxic protein products of tonsillar source.

12. Trans. Assn. Am. Phys., 1913, 28, p. 98.

13. Jour. Exper. Med., 1913, 18, p. 678.

HISTOLOGIC DIFFERENTIATION BY MEANS OF ANILIN STAINS IN ASSOCIATION WITH "REGRESSIVE MORDANTING," WITH ESPECIAL REFERENCE TO ELASTIC TISSUE*

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It is well-known that a number of substances have the power of mordanting stains in animal tissues, and that this result may be brought about, in some cases, by applying the two reagents mixed together, while in others, by the mordants following the dye, or vice versa. In fact, it may be said that a great majority of our staining methods depend on some such action, and that we unquestionably owe our most useful histologic differentiations to a combination of stains and their proper mordants. In view of the foregoing facts, it occurred to me some years ago that it might be possible to employ some of the latter substances before staining and by means of appropriate reagents withdraw them from those structures with which they were only feebly combined, and then secure, by appropriate stains, differentiation of those elements with which they were in more stable union.

As I am aware of no work having been done previously along these lines, I have ventured, for obvious reasons, to call the process "regressive mordanting."

It first became necessary to investigate the action of mordants on tissues fixed in different ways. It was found that elastic tissue exhibits a decided tendency to retain two, at least, different mordanting substances, but formalin fixation is necessary to secure the best results.

It was found that permanganate of potassium and phosphomolybdic acid readily lend themselves to the differentiation of elastic tissue, causing it to become basophilic.

Naturally, the substances used to abstract the mordant from the tissues, with which it is more feebly combined, vary with each reagent employed. With permanganate of potassium it was found that reducing agents readily produce satisfactory results, while, with phosphomolybdic acid, alkalies prove eminently satisfactory.

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For the differentiation of elastic tissue by this process, the following methods were employed:

Fixation in formalin, followed by dehydration, clearing, embedding in paraffin, and cutting processes, and finally fixation of the sections to the slides by means of Mayer's albumin.

After the paraffin is dissolved out of the sections, they are placed in a 1 percent solution of either potassium permanganate or phosphomolybdic acid; in the former, they should remain only a minute or so, as otherwise the tissues become deeply stained, while, in the latter, they may be allowed to soak from a few minutes to twenty-four hours. The sections are then washed in water and, if the potassium permanganate method is employed, placed for a minute in a 1 percent solution of sodium thiosulphate or oxalic acid in water. When the phosphomolybdic acid has been used, they are immersed for about one minute in a solution of 1/10 normal sodium hydroxid, diluted three or four times with water. Unfortunately the strength of the sodium hydroxid solution is often sufficient to cause the sections to come off when Mayer's albumin has been employed for fixing them on the slides; under such circumstances, the tissues may be protected by immersing the sections for an instant in an extremely weak solution of celloidin, and then transferring them to water. This causes the precipitation of a thin film of this substance, which acts as an effective support and permits their being carried through the alkaline solution without injury. This trouble may be likewise obviated by placing the sections in an incubator for twenty-four hours after fixation to the slides.

The section may now be stained, and it will be found that the elastic tissue is very sharply differentiated by basic anilin dyes; this process is extremely rapid, requiring from a few seconds to a minute or so. After the section is completely stained it is usually only necessary to wash it well in alcohol, which removes the excess of stain and dehydrates at the same time, tho, in other instances, differentiation may be only brought about satisfactorily by using slightly acidulated alcohol. The necessity for differentiation largely depends on how thoroughly the mordant has been abstracted from the other structures; should it only be partially withdrawn all of the tissues stain, tho the elastin much more deeply. In this way an excellent counter staining effect is obtained.

Perhaps the most satisfactory of all of the dyes that may be employed is victoria blue in watery solution, in which the sections remain only for a few seconds, are then washed in water, dehydrated, and mounted in the usual way. Where the mordant has not been entirely removed from the other tissues, a particularly satisfactory stain is obtained by allowing the sections to remain for some minutes in a 1 percent solution of victoria blue dissolved in a 1/1,000 aqueous solution of oxalic acid — little or no differentiation being necessary when this method is used. In sections stained in this way it will be found that the elastic tissue fibrils are colored intensely blue, the epithelial structures not so decidedly blue, while the remaining tissues show shades of the same color, depending upon how thoroughly the mordant has been removed, the length of time which the sections remain in the staining solution, and the degree of final differentiation. Another very satisfactory way of employing this method is to stain with carbol fuchsin, particularly after phosphomolybdic acid. In this case the staining process should be somewhat prolonged, requiring 1.5-2 minutes, followed by washing out in alcohol very slightly acidulated with some acid, great care being taken not to carry the differentiation too far. Unfortunately, the carbol fuchsin

method appears to be somewhat capricious, as oftentimes the finest fibrils are not stained. This is due to over-differentiation. Where the method is properly carried out, the results are very effective. I have found it of advantage, after differentiation is completed, to immerse the section for a few moments in the sodium hydroxid solution and then rapidly dehydrate and clear; this is particularly desirable where acid solutions have been used, as otherwise the colors fade. In addition to the stains mentioned, other anilin dyes give excellent results, for instance, thionin, toluidin blue, methylene blue, azure, neutral red, safranin, magdala red, crystal violet, gentian violet, etc. Carbol toluidin blue gives particularly pleasing effects, as does also thionin; when the latter reagent is employed differentiation with acid solutions produces striking results, the tissues generally retaining more or less of a blue color, and the elastic fibrils showing a bluish purple tinge—this being particularly marked after oxalic or tartaric acid. I would again repeat that, after differentiation is completed, all of these stains appear to be somewhat improved, particularly where acid solutions have been employed for the purpose of differentiation, if the sections are immersed, for a few moments, in a solution of sodium hydroxid.

I have been unable to stain the elastic fibrils with either carmin or haematin. Thus it appears to be possible that the chemical constituents which determine basophilism with anilin dyes are not the same as those which unite with and produce staining effects with the two stains referred to. On the other hand, it is also possible that the mordants used have an extraordinary affinity for basic anilin dyes, as well as elastic tissue, and, clinging to the latter, when these methods are used, attracts and firmly retains the former. In other words, it may be simply a reaction between the mordant and the dye which chances to occur in the elastic tissue, as the former substances are held more tenaciously by elastin than by other tissues. It is further noteworthy that epithelial structures stain readily by this method, the protoplasm retaining the dye in a much more pronounced fashion than the nuclei—thus presenting a reaction which would appear to resemble closely that which occurs in the elastic tissue. This effect is so pronounced that it appears in all of the tissues heretofore examined clearly to differentiate epithelial cells from those of mesoblastic origin, and on further trial may prove to be of value in differentiating carcinoma from sarcoma.

As has been before mentioned, counter stains may be employed. Where victoria blue is used, this result may be secured by placing the slide in a watery solution of eosin, picric acid, or acid fuchsin, either before or after coming from the victoria blue bath. Where the tissues are stained with carbol fuchsin, either anilin blue or picric acid gives good results; where the former dye is used the effects are more satisfactory if it precedes the carbol fuchsin; picric acid appears to inter-

fere with the staining in those cases where it is employed before mordanting or just after, and it must, therefore, always follow the basic dye.

The elastic tissue fibrils seem to stain as well, if not better, by Weigert's method after regressive mordanting than without it, while on the other hand they seem to lose almost entirely their capacity to stain by orcein.

These methods clearly show that elastic tissue fibrils may be differentiated into an outer, more intensely staining zone, and an inner zone which, in many instances, fails to take the dye entirely. Where the tissues are properly differentiated, this effect may be observed with the greatest clearness, the outer portion of the elastic tissue fibrils appearing to form what might be called a sheath. This is accentuated by the fact that, in many instances, the fibrils show, for a short distance in either direction from where they are cut in two, a marked increase in the intensity of the peripheral coloration, giving the appearance that the staining solution had penetrated between what might be called the medullary substance and a possible outer sheath. However, I have been unable to definitely determine a physical boundary between the two; in fact the tendency of the stain to show a deeper zone of peripheral intensity of coloration, where differentiation is not carried out so completely, would seem to indicate that the reaction referred to is simply dependent on an increased capacity of the substance of the fibre to retain the stain from within outward.

This method has given extremely gratifying results wherever used, with the exception that both the very fine elastin fibrils and the connective tissue structures, in the outermost layers of the derma, part with the mordant much more readily than do similar structures deeper down in the skin, or even fibrils of elastin of the same size in the walls of the blood-vessels. So readily do these superficial elastin fibrils give up the mordant that they seem to retain it but little, if any, longer than the deeper connective tissues. The result is that where differentiation is carried on to the extent of completely robbing these tissues of the mordanting substance a similar action has likewise occurred in all of the structures comprising the outermost layers of the true skin. Therefore, when it is particularly wished to study the superficial dermal elastin, the differentiating bath should be materially shortened, in which case, however, the deeper collagenous tissues stain almost as intensely as do the elastic tissue fibrils with which they are closely

associated. Elacin, which is usually so abundantly present in the middle layers of the true skin following pellagrademics, does not appear to be altered in its staining reactions by the mordants employed, tho it parts with basic stains more readily than does the normal elastin where the pathologic process is advanced. Therefore, it is generally easy to differentiate the normal from the diseased structures. This is an advantage that these methods have over that of Weigert, since elacin reacts with this stain precisely as does normal elastin.

These results warrant further investigation, and it is my hope that some one else may take up the matter and exhaust its possibilities.

THE EFFECT OF GENTIAN VIOLET ON THE BACILLUS TETANI, TETANUS TOXIN AND CERTAIN LABORATORY ANIMALS *

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We were led through the work of Churchman¹ on the selective action of gentian violet to investigate the devitalization by this dye of toxic cultures of the bacillus tetani intended for immunization of horses.

The use of phenol, or tricresol, or of toluene fails when applied to tetanus cultures, because of the resistance of the spores and the lability of the toxin. Sedimentation and centrifugalization are uncertain and unsafe even when a preservative is added. Berkefeld filtration with or without antiseptic is effective and safe only if extreme care is taken to prevent the spilling of the culture. One of us² has devised an apparatus to facilitate safe separation of the culture from the oil used to exclude air from the medium.

HISTORICAL

As early as 1886 Pfeffer³ showed that certain anilin dyes are harmful to higher plants. The following year Spina,⁴ Roszahegyi,⁵ and Noegerath⁶ demonstrated the reduction of sodium indigo-sulphonate and methylene blue by certain bacteria.

Bekh working with Penzoldt⁷ early proved the truly selective action of certain dyes as did Cornil and Babes,⁸ while Drigalski and Conradi⁹ later emphasized the selective action of crystal violet upon cocci in their well known medium for the isolation of the bacillus typhosus.

Walker and Murray¹⁰ and Vay¹¹ noted morphological changes in organisms grown on media containing dyes and Dreyer, Kriegler and Walker¹² claimed the lethal time to vary in the ratio of the inverse square of the dye concentration.

* Received for publication September 2, 1914.

1. Jour. Exper. Med., 1912, 16, p. 221.

2. Univ. Cal. Pub. in Path., 1913, 11, p. 97.

3. Quoted by Simon and Wood, Am. Jour. Med. Sc., 1914, 147, p. 247.

4. Centralbl. f. Bakteriöl., Abt. I, Orig., 1887, 11, p. 71.

5. Ibid., p. 418.

6. Fortschr. d. Med., 1888, 6, p. 1; quoted by Churchman, Jour. Exper. Med., 1912, 16, p. 221.

7. Arch. f. exper. Path. u. Pharmacol., 1890, 26, p. 310.

8. Les Bacteries, Paris, Third edition, 1890, p. 76.

9. Ztschr. f. Hyg. u. Infektionskrankh., 1902, 39, p. 283.

10. Brit. Med. Jour., 1904, 11, p. 16.

11. Centralbl. f. Bakteriöl., Abt. I, Orig., 1910, 55, p. 193.

12. Jour. Path. and Bacteriol., 1911, 15, p. 134.

As to the mechanism of the germicidal action of dyes, Kriegler¹³ anticipated the conclusions of Simon and Wood¹⁴ in finding diminished lethal action among certain members of the rosanilin and thiazin groups associated with decreasing basicity. May¹⁵ confirmed this for fuchsin and proved the importance of temperature.

Color is an insignificant element in the germicidal behavior of dyes, Simon and Wood having further shown the activity to rest in the basic auxochromic groups of the triamino triphenyl methanes and certain other dyes.

That dyes other than gentian violet possess selective power for bacteria was shown by Churchman,¹⁶ Signorelli,¹⁷ Krumwiede and Pratt,¹⁸ Smythe,¹⁹ Torrey,²⁰ and DeWitt.²¹ Unna's²² success in Gram's staining method with dyes of the para-rosanilin class may be recalled with interest in this regard.

Dye susceptibility may be overcome through adaptation as shown by Shiga.²³ Fitzgerald and Mackintosh,²⁴ while a closely related point is the discovery of susceptible strains by Churchman and Michael²⁵ in a group usually resistant.

THE BACTERIOSTATIC EFFECT OF GENTIAN VIOLET ON THE BACILLUS TETANI

Throughout these experiments, except where specifically noted to the contrary, a filtered saturated solution of Grüber's gentian violet in distilled water was used. This was prepared by the addition of 2 gm. per 100 c.c. distilled water, which is slightly in excess of the amount, 1.75 gm., stated as necessary for saturation by Stitt,²⁶ or 1.5 gm. by Wood.²⁷ Just previous to each test, the portion used was sterilized by placing the test tube container in boiling water several minutes. For use, dilutions such that 1 c.c. contained 0.1 c.c., 0.01 c.c., 0.001 c.c., etc., of saturated solutions were made in sterile 0.85 percent NaCl. These were boiled a few minutes and then cooled. The volume of medium or culture exposed to the action of the dye was 9 c.c. in each case, the addition of 1 c.c. diluted dye yielding the required dilution as noted in the protocols. Controls having no gentian violet were brought to equal volumes with 0.85 percent NaCl.

13. *Centralbl. f. Bacteriol., Abt. I, Orig.*, 1911, 59, p. 481.
14. *Am. Jour. Med. Sc.*, 1914, 147, pp. 247, 524.
15. *Jour. Am. Med. Assn.*, 1912, 58, p. 1174.
16. *Jour. Exper. Med.*, 1913, 17, p. 373; *Ibid.*, 18, p. 187.
17. *Centralbl. f. Bakteriologie, Orig.*, 1912, 64, p. 496.
18. *Proc. New York Path. Soc.*, 1913, N. S. 13, p. 43; *Centralbl. f. Bakteriologie, Abt. I, Orig.*, 1913, 68, p. 562; *Jour. Exper. Med.*, 1914, 19, p. 20; *Ibid.*, p. 501.
19. *Centralbl. f. Bakteriologie, Abt. I, Orig.*, 1913, 71, p. 319.
20. *Jour. Infect. Dis.*, 1913, 13, p. 263.
21. *Ibid.*, 12, p. 68.
22. Quoted by Benians, *Jour. Path. and Bacteriol.*, 1912, 7, p. 199.
23. *Ztschr. f. Immunitätsf., Orig.*, 1913, 18, p. 65.
24. *Proc. Soc. for Exper. Biol. and Med.*, 1913, 10, p. 149.
25. *Jour. Exper. Med.*, 1912, 16, p. 822.
26. *Practical Bacteriology, Blood Work, Parasitology*, Appendix, Philadelphia, 1911.
27. *Chemical and Microscopical Diagnosis*, New York, 1909, p. 683.

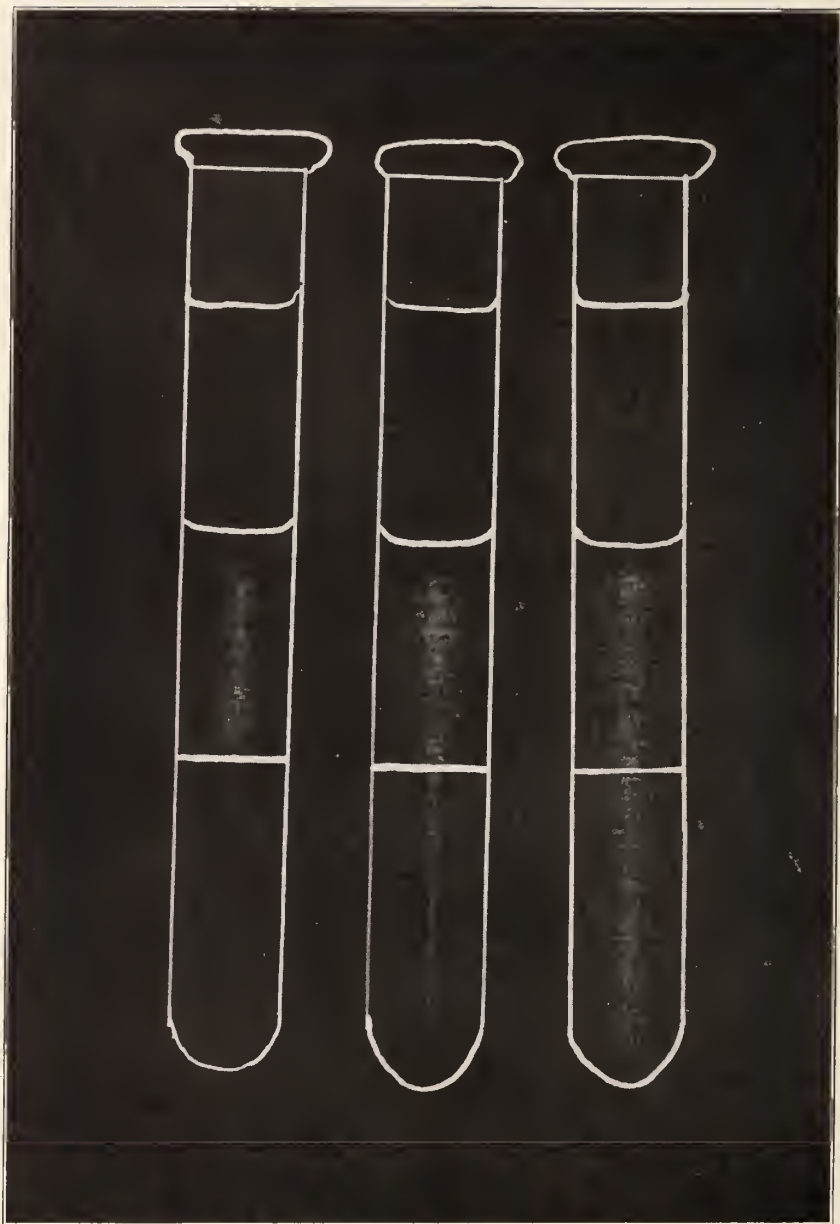


Fig. 1.—Inhibitory effect of gentian violet on the growth of the bacillus tetani in dextrose agar cultures.

A contains 1-1,000 saturated gentian violet, below the paraffin.

B contains 1-10,000 saturated gentian violet, below the paraffin.

C contains no gentian violet.

The strain of the tetanus bacillus used was obtained in April, 1912, from Dr. J. F. Anderson of the Hygienic Laboratory at Washington, D. C.

Experiment 1.—Nine cubic centimeters of sterile neutral 1 percent dextrose agar were placed in each of 3 tubes and the requisite gentian violet solution added, together with a small piece of paraffin. These were boiled 10 to 15 minutes to expel oxygen and melt the paraffin, and were then allowed to cool and harden. Seven to eight cubic centimeters of sterile neutral 1 percent dextrose agar were finally poured into each, leaving the relations within the tubes as in Figure 1. After inoculation from a pure six-day culture of the tetanus bacillus in $MgCO_3$ broth² by deep agar stab through the paraffin, the cultures were incubated at 37 C. for 3 days, with the results apparent in Figure 1. Thus it appeared that the presence of 1 part saturated gentian violet in 10,000 parts neutral dextrose agar is markedly inhibitory while 1 part in 1,000 prevents visible growth.

The next step was to determine if a similar amount of gentian violet in a broth culture of the tetanus bacillus would destroy the organism or inhibit its growth on subculture. Preliminary experiments had indicated that a larger quantity of gentian violet was necessary to prevent positive subcultures from tetanus emulsions than to inhibit growth when incorporated in the medium. It is not surprising that such is the case in view of the larger number of organisms to be acted upon in the latter instance. Churchman¹ has mentioned that inhibition is more constant when the dye is incorporated in media than when applied directly to the bacterial bodies.

Krumwiede and Pratt¹⁸ have shown that the reaction is quantitative for certain organisms, an observation in accord with our own on the tetanus bacillus as well as on the anthrax bacillus, staphylococcus albus and aureus, and the gonococcus. With these, and Fitzgerald and Mackintosh,²⁴ we have found the presence of serum albumin to partially inhibit the bacteriostatic effect of the dye.

The following experiment was made on the same day with the tetanus suspension, gentian violet dilutions, and dextrose agar used in Experiment 1. The results are therefore comparable:

Experiment 2.—Nine cubic centimeters of suspension were placed in each of 4 test tubes and diluted gentian violet added in each instance to produce a total volume of 10 c.c. with a gentian violet content as noted in the protocol. The mixtures were then incubated at 37 C. for one and one-half hours and shake cultures in neutral 1 percent dextrose agar prepared. A second series of cultures was made after twenty-four hours' incubation, followed in each instance by seventy-two hours' residence in the thermostat at 37 C. Readings were made after seventy-two hours' incubation of the cultures.

Ratio of Saturated Gentian Violet to Mixture	Plants Made After One and One-Half Hours	Plants Made After 24 Hours' Incubation
1-100	Few distinct punctiform colonies	None
1-1,000	Good, diffused slightly	Fair
1-10,000	Marked, very diffuse	Good
None	Marked, very diffuse	Marked

No aerobic growth was observed in any tube, thus indicating freedom from aerobic contamination. The filtrate of a portion of the culture produced severe tetanus in a guinea-pig, weighing 312 gm., in 4 days after the inoculation of 0.00005 c.c.

These experiments justify the conclusion that whereas 1 part saturated gentian violet solution in 1,000 parts of neutral 1 percent dextrose agar prevents visible growth of the tetanus bacillus and 1 part in 10,000 is almost completely inhibitive, 1 part saturated gentian violet in 1,000 parts of 1 percent dextrose broth culture of the tetanus bacillus during contact at 37 C. for twenty-four hours does not preclude a positive subculture on 1 percent dextrose agar, while 1 part in 10,000 under similar conditions yields a subculture as luxuriant as in the case of the control test with no gentian violet.

Experiment 3.—A week old culture of the tetanus bacillus in dextrose broth was divided between two tubes in equal quantities of 9 c.c. each. To Tube A was added 1 c.c. of 1-10 saturated solution of gentian violet in 0.85 percent NaCl. To Tube B was added 1 c.c. 0.85 percent NaCl. Both were incubated 24 hours at 37 C. and were then washed free from toxin by five successive centrifugalizations in 0.85 percent NaCl. The toxic filtrate from the unused portion of culture being proved by test to contain about 10,000 M. L. D. per c.c., five repeated washings may be considered sufficient to remove all but less than 1 M.L.D. of toxin from the bacteria. The sediment from each tube was re-emulsified in 0.85 percent NaCl and injected subcutaneously into a guinea-pig. Aerobic cultures from each sediment showed the presence of a contaminating gram-positive coccus.

As a result of this test both guinea-pigs died from tetanus in 27 and 20 hours, respectively.

Experiment 4.—A new, week-old culture of the tetanus bacillus in dextrose broth was tested for purity by microscopic examination and successfully barren aerobic subculture on an agar slant. It was distributed in quantities of 9 c.c. each in sterile test tubes and 1 c.c. of saturated gentian violet dilutions in 0.85 percent salt solution added so that the final volume of 10 c.c. in each tube contained the proportion of saturated gentian violet solution indicated in Table 1. The mixtures were then incubated for 24 hours at 37 C. and subcultures made by stab to deep 1 percent dextrose agar.

The mixtures, containing respectively 1-10, 1-100, and 1-200, were centrifugalized, the supernatant fluid decanted, and the sediment washed by centrifugalization seven successive times in 0.85 percent NaCl. Each was then injected subcutaneously into a guinea-pig. The result shows that the bacilli may be deemed dead after treatment with 1-10 saturated gentian violet at 37 C. for

24 hours. But tho they were unable to grow in deep dextrose agar after treatment with 1-600 (and even less) saturated gentian violet for a similar period, the injection reproduced the disease in the guinea-pig after exposure to a dilution of 1-100 parts saturated gentian violet solution.

Exposure in the tube containing 1 part in 10, however, resulted in the destruction of the organisms.

TABLE 1
THE BACTERIO-STATIC ACTION OF GENTIAN VIOLET ON THE *BACILLUS TETANI*

Experiment	Tube	Final Proportion of Saturated Gentian Violet in Mixture*	Result After Incubation of Sub-culture to Deep Dextrose Agar	Symptoms Following Injection of Mixture into Guinea-Pig†									
				Days									
				1	2	3	4	5	6	7	8	9	10
3	A	1-100	d									
	B	None	+	+								
4	A	1-10	Sterile	—	—	—	—	—	—	—	—	—	—
	B	1-100	Sterile	—	—	a	c	d	d	d	d	d	c
	C	1-200	Sterile	—	—		+						
	D	1-400	B. tetani										
	E	1-600	Sterile										
	F	1-800	Sterile										
	G	1-1000	Sterile										
	H	1-2000	Sterile										
	I	None	B. tetani plus contamination										

* Incubated 24 hours at 37 C.

† — = none; a = slight—i. e. just perceptible; b = marked—pig regains feet when placed on back; c = severe—pig regains feet with difficulty only; d = very severe—pig cannot regain feet; + = death.

The weight of the guinea-pigs ranged from 235-279 gm.

It must be emphasized here that inoculations of media were made with a platinum loop carrying an extremely small number of bacterial bodies as compared to a 5 c.c. syringe. It is not conclusive from this, then, that the organisms successfully propagating in the animal body might not have grown successfully also in the culture tube. In fact, the irregular appearance of a positive culture at 1-400 indicates that the amount of material used to inoculate the culture media was scarcely sufficient to be representative. Possible error then would consist in assuming negative cultures as proof of the death of the organisms while the result of the animal test shows that more than 1 part gentian violet solution to 100 parts culture is necessary to destroy the virulence of such emulsions. On the other hand, we are not justified from these experiments in assuming the presence of living organisms in even washed cultures which produce tetanus, since there may be sufficient toxin adherent to the possibly dead bodies to cause symptoms and death from tetanus.

THE EFFECT OF GENTIAN VIOLET ON TETANUS TOXIN

The determination of the effect of gentian violet on the toxin of tetanus presents less difficulties than the preceding phase of the subject since the bacillus and its spores may be eliminated by Berkefeld filtration of a toxic culture.

Experiment 5.—In this test, use was made of a solution of the Hygienic Laboratory test toxin "D" in 0.85 percent NaCl containing 0.0006 gm. dried tetanus toxin per c.c. To 9.9 c.c. in test tube "A" was added 0.1 c.c. saturated aqueous gentian violet, thus making a dilution of 1-100. To a similar amount in Tube "B" was added 0.1 c.c. distilled water. Both were incubated one-half hour at 37 C. Dilutions from each were made by adding 1 c.c. to 99 c.c. 0.85 percent NaCl solution. One cubic centimeter of this dilution should contain approximately 1 M. L. D. and this amount was injected from each into separate guinea-pigs. There was no perceptible difference between the results in these animals, both of which developed mild tetanus on the second day, very severe tetanus on the third day, and died within the same hour late on the third day after injection.

Experiment 6.—To 9 c.c. tetanus culture filtrate was added 1 c.c. saturated aqueous gentian violet in Tube "A." Similarly to 9 c.c. in Tube "B" was added 1 c.c. 0.85 percent NaCl. Both mixtures were incubated at 37 C. for 24 hours and then each diluted to 1-1,000. One cubic centimeter of each dilution was injected subcutaneously into a guinea-pig with the result that the guinea-pig receiving the gentian violet treated toxin did not show symptoms of tetanus in 11 days, while the control developed tetanus on the second day, and died of the disease on the sixth day after injection.

It may be noticed in these preliminary experiments that there are three important factors at variance, namely, the strength of gentian violet, the strength of toxin, and the time of exposure. Another experiment was therefore done in order to ascertain the effect of variation in the strength of gentian violet alone.

Experiment 7.—To each of 4 test tubes containing 9 c.c. fresh tetanus toxin was added 1 c.c. gentian violet dilution in 0.85 percent NaCl such that the mixtures contained respectively 1-10, 1-100, 1-1,000, and no gentian violet in solution. These were incubated at 37 C. for 24 hours. Dilutions were then made in 0.85 percent NaCl such that 1 c.c. contained approximately 1 M. L. D., i. e., 0.00012 c.c. tetanus toxin, and this amount was injected subcutaneously into a normal guinea-pig. All of these guinea-pigs died of tetanus, excepting the one receiving toxin treated with gentian violet 1-10, this animal surviving without symptoms of any sort. Table 2 is a summary of Experiments 5, 6, and 7.

Since a dilution of 1-10 is the least amount, within the limits tested, of gentian violet which suffices to kill tetanus spores, it is obviously impossible to devitalize tetanus cultures by this method and at the same time retain the full toxicity of the filtrate. We are now interested in determining how complete the destruction of toxin might be in the presence of this strength of the dye.

TABLE 2
THE EFFECT OF GENTIAN VIOLET ON TETANUS TOXIN

Mixture of Gentian Violet with Tetanus Toxin					Symptoms Following Subcutaneous Injection of 1 M. L. D.*											
Experi- ment	Tube	Proportion of Saturated Aqueous Gentian Violet Solution	Potency of Tetanus Toxin per c.c.	Time of Incuba- tion of Mixture in Hours	Guinea-Pig Weight in Grams	Days										
						1	2	3	4	5	6	7	8	9	10	
5	A	1-100	100 M. L. D.	0.5	410	..	a	+								
	B	None	100 M. L. D.	0.5	455	..	a	+								
6	A	1-10	1000 M. L. D.	24	398	—	a	—	c	—	+	—	—	—	—	—
	B	None	1000 M. L. D.	24	392	?	—	—	—	—	—	—	—	—	—	—
7	A	1-10	8000 M. L. D.	24	330	—	—	—	—	—	—	—	—	—	—	—
	B	1-100	8000 M. L. D.	24	335	—	—	—	—	—	—	—	—	—	—	—
	C	1-1000	8000 M. L. D.	24	340	—	—	—	—	—	—	—	—	—	—	—
	D	None	8000 M. L. D.	24	345	—	a	a	b	d	d	+	+	+	+	+

*— = none; a = slight—i. e., just perceptible; b = marked—pig regains feet when placed on back; c = severe—pig regains feet with difficulty only; d = very severe—pig cannot regain feet; + = death.

Experiment 8.—A fresh saturated solution of gentian violet was made in 0.85 percent NaCl. One cubic centimeter of this solution was added to 9 c.c. of a potent tetanus toxin, and at the same time, 1 c.c. of 0.85 percent NaCl was added to another tube containing 9 c.c. of this toxin. These mixtures were placed in the thermostat room at 37 C. for 24 hours. Dilutions in 0.85 percent NaCl, ranging from 0.2 to 0.0002 c.c. toxin per c.c. were then made, as outlined in the protocol below, and guinea-pigs injected subcutaneously with 1 c.c. of each dilution. Similarly, a guinea-pig received 0.0002 c.c. of the toxin to which only the salt solution had been added in 1 c.c. of 0.85 percent NaCl.

TABLE 3
PROPORTION OF TETANUS TOXIN DESTROYED BY CONTACT WITH 10 PERCENT SATURATED GENTIAN VIOLET

Toxin Content of 1 c.c. Diluted Mixture	Weight of Guinea-Pig	Symptoms after Subcutaneous Injection of 1 c.c.*									
		Days									
		1	2	3	4	5	6	7	8	9	10
0.2	480	—	b	d	+						
0.02	495	—	a	b	a	b	c	b	?	b	b
0.002	485	—	—	?	a	a	—	b	?	a	a
0.0002	475†	—	—	—	—	—	—	—	+		
0.0002 (Control)	475	+									

* — = none; a = slight—i. e., just perceptible; b = marked—pig regains feet when placed on back; c = severe—pig regains feet with difficulty only; d = very severe—pig cannot regain feet; + = death.

† Death not due to tetanus.

The results, as shown in Table 3, indicate that there is almost total, tho not complete, destruction of toxicity under the influence of 1 part saturated gentian violet solution to 9 parts tetanus toxin, when incubated twenty-four hours at 37 C. It may be noted here that the toxin was very powerful; there must have been many times the M.L.D. given to the control guinea-pig in 0.0002 c.c. untreated toxin. Yet a dose ten times as large of treated toxin failed to cause more than slight symptoms of tetanus during a period of ten days of observation, a dose one hundred times as large gave only moderate symptoms, and a thousand times as much was required to kill the injected animal with tetanus, which it did upon the fourth day.

Assuming that 0.0002 c.c. of the toxin contained only 1 M.L.D. for the test animal, 0.02 c.c. of the toxin would contain 100 M.L.D., and death should occur in a few hours after injection. The symptoms of the animal receiving this amount of treated toxin, however, indicate the presence of less than 1 M.L.D. of potent toxin, which signifies

the destruction of over 99 percent of the toxin by the action of 0.1 saturated solution of gentian violet. The guinea-pig which received 0.0002 c.c. treated toxin died upon the eighth day, but not of tetanus. The cause of death was not ascertained.

These experiments on tetanus toxin yielded such regular results that we were surprised that Churchman²⁸ found gentian violet to give inconstant results with tetanus toxin. Dr. Meyer²⁹ considers adsorption the basis of the destructive action of the dye, a view which finds affirmation in our experiments.

THE TOLERANCE OF RABBITS AND GUINEA-PIGS TO GENTIAN VIOLET

The purpose of recording the tests below is to demonstrate the surprisingly slight toxicity of injections of gentian violet for small animals.

Subcutaneous injections.—Following are the details of a test made upon three guinea-pigs by a single injection each of the dye subcutaneously. A saturated solution of Grüber's gentian violet in distilled water was used. Immediately previous to use, it and the dilutions made therefrom, in 0.85 percent NaCl, were sterilized by boiling. On cooling, the injections were made in each instance with the usual aseptic precautions.

Guinea-pig A, weight 592 gm., was injected subcutaneously with 1 c.c. saturated aqueous gentian violet. In 24 hours a marked induration appeared. In four days there was an ulcerated phlegmon involving necrosis of the inguinal glands. This animal recovered fully after daily treatment for two weeks with 2 percent tricesol solution to prevent infection. During this period the odor from the wound was very disagreeable. The animal was in splendid condition two and one-half months later.

Guinea-pig B, weight 440 gm., was injected subcutaneously with 1 c.c. of 10 percent saturated gentian violet in 0.85 percent NaCl. In five days a slight induration was noticed. In nine days there was a small open ulcer. This pig recovered fully in two weeks without treatment, and remained in good condition for at least two and one-half months.

Guinea-pig C, weight 250 gm., was injected subcutaneously with 0.5 c.c. of 10 percent saturated gentian violet in 0.85 percent NaCl. There were no visible effects or lesions and the animal was released from observation after three weeks.

A number of tests not noted herein had indicated that 10 c.c. of 1 percent saturated gentian violet in 0.85 percent NaCl has no apparent effect in subcutaneous injections of rabbits. In order to determine the result of repeated injections two animals were placed in a cage, the intention being to inject the heavier one, designated Rabbit A, weight 2,400 gm., subcutaneously every few days with 10 c.c. of a sterile 1 percent saturated solution of gentian violet in 0.85 percent NaCl. Rabbit B, weight 2,150 gm., was to be observed without

28. Jour. Am. Med. Assn., 1913, 61, p. 302; Proc. Soc. for Exper. Biol. and Med., 1914, 11, p. 54.

29. Personal communication.

injection as a control upon Rabbit A. After four days, it was found that both rabbits had lost approximately 15 and 10 percent in weight, respectively. Inquiry indicated fighting as a probable cause and they were therefore separated, with a rapid increase in weight to about normal. At this time the plan of experiment was changed so that Rabbit B received 10 c.c. of sterile 0.85 percent NaCl subcutaneously, at the same time that A was treated with 10 c.c. of 1 percent gentian violet solution. In all, A received five 10 c.c. doses of 1 percent saturated gentian violet solution; B received three 10 c.c. doses of 0.85 percent NaCl. Table 4 shows that the relative weights of these animals had interchanged at the end of one month altho there was no sign of disturbance of function in either, except slight swellings at the site of inoculation in Rabbit A after the second and third injections. These disappeared rapidly in about thirty-six hours.

TABLE 4

COMPARATIVE WEIGHTS OF RABBITS RECEIVING SUBCUTANEOUS INJECTIONS OF GENTIAN VIOLET AND 0.85 PERCENT SALT SOLUTION

Day of Observation	Rabbit A		Rabbit B	
	Treatment	Weight in Grams	Treatment	Weight in Grams
1	10 c.c. 1 percent satur. gentian violet s. c.	<i>2400</i>	None	2150
3	10 c.c. 1 percent satur. gentian violet s. c.	<i>2260</i>	None	2120
5*	10 c.c. 1 percent satur. gentian violet s. c.	<i>2025</i>	10 c.c. 0.85 percent NaCl	1940
8	10 c.c. 1 percent satur. gentian violet s. c.	<i>2360</i>	10 c.c. 0.85 percent NaCl	2260
10	10 c.c. 1 percent satur. gentian violet s. c.	<i>2350</i>	10 c.c. 0.85 percent NaCl	2200
14	10 c.c. 1 percent satur. gentian violet s. c.	<i>2280</i>	10 c.c. 0.85 percent NaCl	2180
16	10 c.c. 1 percent satur. gentian violet s. c.	<i>2200</i>	10 c.c. 0.85 percent NaCl	2150
18	10 c.c. 1 percent satur. gentian violet s. c.	<i>2325</i>	10 c.c. 0.85 percent NaCl	2270
21	10 c.c. 1 percent satur. gentian violet s. c.	<i>2140</i>	10 c.c. 0.85 percent NaCl	<i>2290</i>
23	10 c.c. 1 percent satur. gentian violet s. c.	<i>2160</i>	10 c.c. 0.85 percent NaCl	<i>2275</i>
25	10 c.c. 1 percent satur. gentian violet s. c.	<i>2200</i>	10 c.c. 0.85 percent NaCl	<i>2345</i>
29	10 c.c. 1 percent satur. gentian violet s. c.	<i>2240</i>	10 c.c. 0.85 percent NaCl	<i>2350</i>

* Animals previously in one cage; now separated.

The weight of the heavier animal at each observation is printed in *italic* to indicate the fact more clearly.

These tests confirm the observations of Churchman and Herz³⁰ as to the possibility of treating animals with considerable amounts of dye without material harm. We have gained the idea that subcutaneous injections are more or less irritating locally, but not fatal in comparatively large repeated doses. In some instances certain glands, near the site of injection, appeared particularly susceptible.

Intravenous injections.—Rabbit C, half-grown, was subjected to the intravenous inoculation of 1 c.c. saturated gentian violet in distilled water without visible symptoms of any sort up to the eighth day. At that time, a second intravenous injection was attempted but most of the stain was injected into the subcutaneous tissues near the base of the ear. The following day the jaw and ear were badly swollen and five days following the injection the animal was killed because of its obvious suffering. On necropsy the viscera were normal with the exception of the intestines, which were cyanotic. The ear and face were much swollen on the side of the injection and one section displayed the parotid gland neatly outlined in pale blue with the connective tissues surrounding it presenting a jelly-like appearance through the presence of edema fluid. The ear was edematous and thickened, but not markedly stained.

Rabbit D, an adult, weight 2,590 gm., a month previous subjected to a non-virulent subcutaneous injection of the staphylococcus aureus, was inoculated intravenously with 1 c.c. saturated aqueous solution of gentian violet on three successive days. These injections were successful excepting the last, of which approximately 0.1 c.c. escaped into the subcutaneous tissue of the ear. There were no signs of discomfort of the animal nor changes in the tissues until the following morning when this ear was found slightly edematous and paralyzed. The rabbit was dead the next day.

Necropsy revealed a marked subcutaneous edema of the ear and a markedly hemorrhagic condition of the lungs; all of the other organs appeared normal, particularly the heart, liver, stomach, spleen, kidneys, intestines, mesentery, and peritoneum; heart blood cultures gave a gram-negative coliform bacillus.

Rabbit E, an adult, which had been used before, was injected intravenously with 1 c.c. saturated aqueous gentian violet. There was a slight swelling and paralysis of the ear, due, no doubt, to faulty technic of injection, but the animal survived until the sixteenth day, when it was found dead. Necropsy proved death due to a staphylococcic abscess of the pleural cavity.

At the same time a similar animal, Rabbit F, was injected intravenously with 5 c.c. of a saturated aqueous solution of gentian violet. The lips, ears, tongue, and eyelids became intensely purple in a few minutes followed immediately by stupor, loss of motor power, and motor reflexes. The animal quickly became unconscious and during a paroxysm of choking, in which the urine was voided, it died, a period of about fifteen minutes having elapsed since the injection. Necropsy showed the stain distributed throughout the body and especially notable in visceral organs.

Of interest in this connection is a series of observations upon another rabbit. This animal, Rabbit G, had previously been injected intravenously, without observable effect, with 2 c.c. saturated gentian violet in 0.85 per cent NaCl. After the outcome of the injections of Rabbits E and F, 5 c.c. of a sterile saturated solution of gentian violet in 0.85 percent NaCl, were injected intra-

venously, a period of ten weeks having elapsed since the first injection. No observable effects followed this treatment. A week later, 10 c.c. were injected intravenously, using, in all intravenous injections, the marginal vein of the ear. Again there were no indications of discomfort or illness.

We desire to note here that the solution used for the last two injections was several months old, but if the age of the solution is a factor in the toxicity of gentian violet, it did not operate in the case of the saturated solution of gentian violet in 0.85 percent NaCl which was made at about the same time as the aqueous solution and kept under the same conditions of room temperature and diffuse light. For when 5 c.c. of the latter, i e., the old aqueous solution, were injected intravenously two weeks later, the rabbit, now very large, weighing 2,700 gm., was dead in three minutes after a succession of symptoms essentially like those described for Rabbit F. Immediate necropsy showed the mucosa and viscera deeply stained, but, altho the animal was very fat, there was no adsorption of the dye by the fat, which stood out prominently upon the reddish purple background of the stained tissues.

That 5 c.c., or even 10 c.c., distilled water injected intravenously does not cause sudden death was easily demonstrated in Rabbit H, weight 1,240 gm., which was so treated. There was no evident discomfort, altho the weight of this rabbit was less than half that of Rabbit G.

Further comparison between aqueous solutions and salt solutions of gentian violet should be made. Our experience indicates a change in technic from saturated solutions to solutions containing definite proportions, less than saturation, of solid dye.

Intravenous injections of gentian violet are thus more toxic than subcutaneous inoculations, the dye being quickly diffused and sudden death occurring when 5 c.c. or more of aqueous solution were injected in the ear vein.

SUMMARY

Gentian violet distinctly inhibits the growth of the tetanus bacillus when 1 part saturated solution is added to 10,000 of dextrose agar. In dextrose broth, however, exposure for twenty-four hours at 37 C., at this concentration, has no perceptible effect on subcultures, tho cultures failed from broth containing 1 part in 100. The organisms are not destroyed, however, as can be shown by animal inoculations. One part saturated solution in ten of broth destroys the virulence of a toxic culture.

At this concentration more than 99 percent of the toxin is destroyed by contact at 37 C. for twenty-four hours. One part in one hundred has no perceptible effect.

The necessity of using more than a 1 percent saturated solution for devitalization precludes the employment of gentian violet for the practical purpose attempted through excessive soiling of laboratory ware. Within the limits tested, the toxin appears to be destroyed under practically the same conditions as the spores.

Guinea-pigs and rabbits withstand considerable injections of gentian violet in 0.85 percent NaCl. Distilled water solutions appear to be more toxic than those in salt solution, and subcutaneous injections less than intravenous injections.

THE PRODUCTION OF ACID BY THE BACILLUS COLI GROUP*

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The year 1885 marks the beginning of the study of the bacteria of the intestinal tract in relation to their action on the various carbohydrates. In that year, Buchner, working with his "Darmbacillus G," which, in all probability, was a member of the bacillus coli group, found that growth in a medium, consisting of meat infusion, peptone, and sugar, was accompanied by the formation of acid and gas. The acid was demonstrated by the addition of litmus to the medium. The production of acid and gas, according to Buchner, was due to the breaking down, or decomposition, of the sugar brought about by the action of the bacteria. The evolved gas was found to be carbon dioxide, and the acids were defined as members of the fatty acid series. The work of Buchner may be considered as the starting point of all the work done on the relation of bacteria to the decomposition of carbohydrates with the production of acid and gas.

In connection with the production of acid in carbohydrate solutions by bacteria, progress has been particularly active along two distinct lines: (1) The differentiation of the colon group from the typhoid group and other alkali producers, and (2) the classification of the bacillus coli group, according to the ability of its members to ferment the various carbohydrates.

Petruschky's litmus whey medium, Beijerinck's calcium carbonate medium, Wurty's litmus lactose agar, Kaufmann's jequirity solution, and Hanna's proteid medium were important steps in the investigations along the former line. Among the workers of this period, who have contributed to our knowledge of carbohydrate fermentation, are Beginsky (1888), Lembke (1896), Von Sommeruga (1892), Capaldi and Proskauer (1897), Zielleyky (1902) and Segin (1903). These results were augmented by Theobald Smith by use of the fermentation tube.

Beginning with the work of Durham in 1900, nearly all the investigations along the line of fermentation of carbohydrates by the bacillus coli group have been done with the idea of classifying the members of the group by means of their fermentative reactions on the various carbohydrates. The work of Dur-

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ham has been extended and augmented by the notable researches of Houston (1902), MacConkey (1905), Winslow and Walker (1907), Graham Smith (1909), and Jackson (1911).

In spite of the importance of this fermentative reaction, however, there are many of the finer points of technic which have never been fully and conclusively worked out. Acid production, like all other biochemical reactions, is a function of the reacting organism, the substance decomposed, the end products formed, the time, the temperature, and all conditions which may check or favor the vital process. In the present investigation, I have attempted to isolate these various factors and study them, one by one, with the idea of gaining a clear idea of the quantitative significance of each. No startlingly novel conclusions could be expected, but it is hoped that the data obtained may furnish a surer basis for comparative studies of fermentative power in carbohydrate media than has been available in the past.

ORGANISMS USED IN THE INVESTIGATION

The organisms used in this work were all members of the bacillus coli group. Altho the identification of this group has been a subject of dispute for over a quarter of a century, it is now generally conceded that the following characteristics may be considered criteria of membership: (1) Short bacillus with rounded ends; (2) gram-negative; (3) non-liquefaction of gelatin in 16 days; (4) fermentation of dextrose and lactose with the production of acid and gas; (5) non-spore-forming; (6) facultative anaerobe; (7) gas in lactose-peptone-bile; (8) grayish white growth on agar at 20 C. and 37 C.

Other characteristic properties of this group, such as the reduction of nitrates to nitrites, production of indol, motility, and style of growth on various media, are valuable, if at all, merely for establishing minor subdivisions.

The organisms used in this investigation all conformed to the general characteristics of the bacillus coli group, as noted. In the early part of the work, no particular attention was paid to the subdivisions of the group, and, as a result, organisms were used which were identified only by the general group characteristics. As the work developed and the number of carbohydrates in use increased, the subdivisions of the group began to manifest themselves. A clean-cut differentiation into various species was obtainable by the production

or non-production of acid in the carbohydrate solutions. As the writer had no dulcete at hand, a subdivision of the group into species, as recommended by Jackson, was impossible, and, as a result, all the strains used throughout the work have been designated as the bacillus coli, altho it is certain that a number of species were present. As far as possible, those organisms which gave similar fermentative reactions have been grouped in the same table.

The cultures were obtained from two sources; either from oysters grown in contaminated water, or directly from feces. The oysters, from which the organisms were isolated, were taken from 242 different stations in Narragansett Bay during an investigation to determine the amount of pollution of the oyster-beds of the State of Rhode Island. The area from which the oysters were taken included localities, varying from those extremely polluted near sewer outlets to the less polluted areas, which were sometimes coli-positive and other times coli-negative.

One series of the cultures, isolated from feces, was obtained from normal stools of people in and about the laboratory at Brown University, while the other series was obtained from the stools of Italian immigrants, quarantined aboard the *S. S. Roma*, who were undergoing examinations for the cholera vibrio. These two series were kept entirely distinct and will be so designated throughout the work.

METHODS

Preparation of Culture Media.—The media were prepared after the methods proposed in the Report of the Committee on Standard Methods of Water Analysis to the Laboratory Section of the American Public Health Association, January 5, 1905, Liebig's meat extract was used, 3 grams to the liter. Ease of preparation rendered it more suitable for the work of this kind than a medium made up with meat. Great care was taken in the preparation of the media to make all the lots as uniform as possible. For this reason, all the constituents were taken from the same lot of materials during the entire course of the experiments.

The carbohydrate solutions were made by the addition of 1 percent of the various carbohydrates to neutral nutrient broth. Twenty-five cubic centimeters of the broth were placed in regular laboratory test tubes of large size, and sterilized on three successive days in streaming steam at 100 C.

During the course of the experiments, the production of acid was determined in the following carbohydrates:

I. I. Monosaccharids:

A. Hexoses.

1. Dextrose (Merck).
2. Galactose (Merck).
3. Levulose (Kahlbaum).

B. Pentoses.

1. Arabinose (Kahlbaum).
2. Xylose (Kahlbaum).

II. Disaccharids:

1. Lactose (Merck).
2. Maltose (Merck).
3. Saccharose (Merck).

III. Trisaccharid:

1. Raffinose (Kahlbaum).

IV. Hexatomic alcohols:

1. Iso-dulcite (Kahlbaum).
2. Mannite (Merck).

All cultures used in the determination of the production of acid in the carbohydrate solutions were grown at 37 C. for 24 hours, unless otherwise stated. The titrations were made with N/20 sodium hydroxid at the twenty-fourth hour, and results tabulated in direct percentages of normal sodium hydroxid.

Method of determining amount of acid produced.—From a twenty-four-hour agar slant culture of the organism, which had been identified as a member of the bacillus coli group, inoculations were made into peptone solutions, which were incubated for 24 hours at 37 C. At the end of 24 hours, the tubes of carbohydrate solution were inoculated by the addition of 0.5 c.c. of the twenty-four-hour peptone culture. By the addition of a definite amount of the twenty-four-hour culture, more consistent results were obtained than by the direct inoculation from the agar slant culture.

The inoculated carbohydrate solutions were incubated for 24 hours and were then ready for titration. The cultures were titrated as soon as possible after their removal from the incubator.¹

Five cubic centimeters of the culture and 45 c.c. of distilled water were placed in a casserole and boiled briskly for one minute. One cubic centimeter of phenolphthalien, which consisted of 5 gm. of the commercial salt dissolved in one liter of 50 percent alcohol, was added as an indicator. Titrations were made into the hot solution until a faint, but permanent, pink color was obtained with N/20 sodium hydroxid solution.

THE RELATION OF TEMPERATURE TO THE AMOUNT OF ACID PRODUCED

Twenty-five cubic centimeters of the various sterilized carbohydrate solutions were inoculated with 0.5 c.c. of a twenty-four-hour peptone culture of the identified organisms. The cultures and their controls were kept for 24 hours at the following temperatures: Ice-box temperature, 3 C.; room temperature, 16 C.; incubator temperature, 28 C.; incubator temperature, 37 C.; incubator temperature, 45 C.

At the end of the twenty-fourth hour the cultures and their controls were titrated as soon as accuracy would allow. All titrations were made with N/20 sodium hydroxid, and all results are expressed in direct percentages of normal sodium hydroxid.

The results obtained in these experiments show that cultures of the bacillus coli group, grown at a temperature of 37 C., tend to produce more acid in carbohydrate solutions, in a given time, than cultures grown at other temperatures. Cultures grown at 3 C. show almost no acid production in 24 hours. As we approach 37 C., either from below

1. The method of titration followed was the one outlined in Standard Methods of Water Analysis, 1905, p. 106.

TABLE 1
AMOUNT OF ACID, IN PERCENT NORMAL, PRODUCED AT DIFFERENT TEMPERATURES BY *BACILLUS COLI*, ISOLATED FROM FECES

Temperature	Percentage of Acid with Dextrose	Percentage of Acid with Lactose	Percentage of Acid with Levulose	Percentage of Acid with Galactose	Percentage of Acid with Maltose	Percentage of Acid with Xylose	Percentage of Acid with Arabinose	Percentage of Acid with Mannite	Percentage of Acid with Isodulcitol	Percentage of Acid with Control
3 C.	0	0	0	0	0	0	0	0	0	0
16 C.	0.6	0.3	0.7	0.3	0.3	0.3	0.3	0.3	0.2	0
28 C.	2.0	1.7	2.1	1.3	1.6	1.8	1.7	1.7	1.7	0
37 C.	2.3	1.9	2.3	1.9	1.9	2.0	2.0	2.0	2.2	0
45 C.	1.3	1.0	1.5	0.9	0.3	1.3	1.2	1.0	0.2	0

The results are the average of the titrations of two cultures.

TABLE 2
AMOUNT OF ACID, IN PERCENT NORMAL, PRODUCED AT DIFFERENT TEMPERATURES BY THE *BACILLUS COLI*, ISOLATED FROM OYSTERS

Temperature	Percentage of Acid with Dextrose	Percentage of Acid with Saccharose	Percentage of Acid with Levulose	Percentage of Acid with Galactose	Percentage of Acid with Maltose	Percentage of Acid with Raffinose	Percentage of Acid with Xylose	Percentage of Acid with Arabinose	Percentage of Acid with Mannite	Percentage of Acid with Isodulcitol	Percentage of Acid with Control
3 C.	0	0	0	0	0	0	0	0	0	0	0
16 C.	0.1	0.1	0.6	0.2	0.3	0.2	0.3	0.3	0.3	0.2	0
28 C.	1.7	1.4	2.1	1.2	1.6	0.9	1.6	1.6	1.4	0.8	0
37 C.	2.0	1.4	2.3	1.8	1.9	1.5	1.9	2.0	1.9	2.0	0
45 C.	0.7	0.3	1.3	0.8	0.2	0.2	1.2	1.0	0.9	0.2	0

The results are the average of the titrations of two cultures.

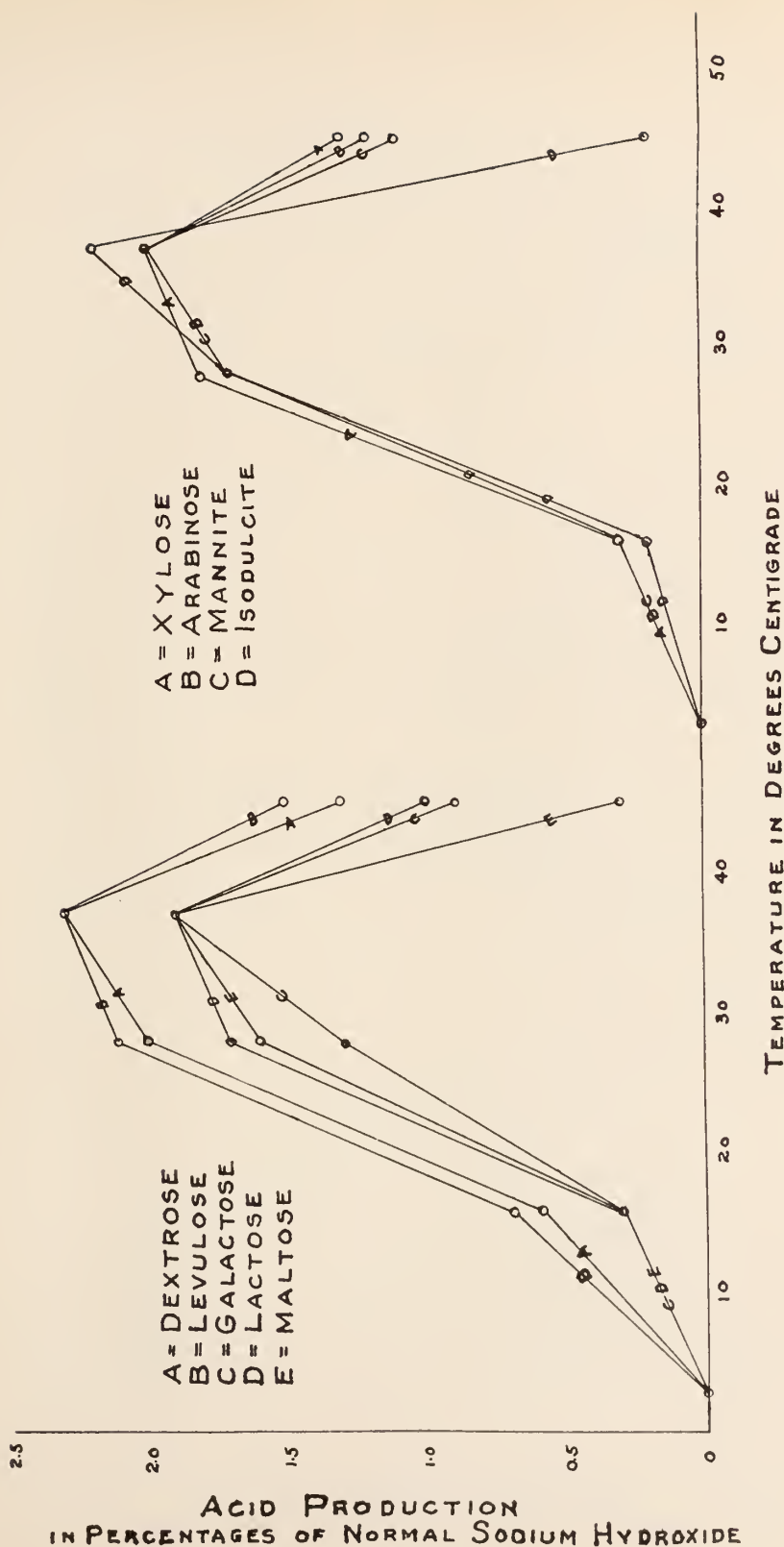


Chart 1.—The relation of temperature to the amount of acid produced by the bacillus coli, isolated from feces.

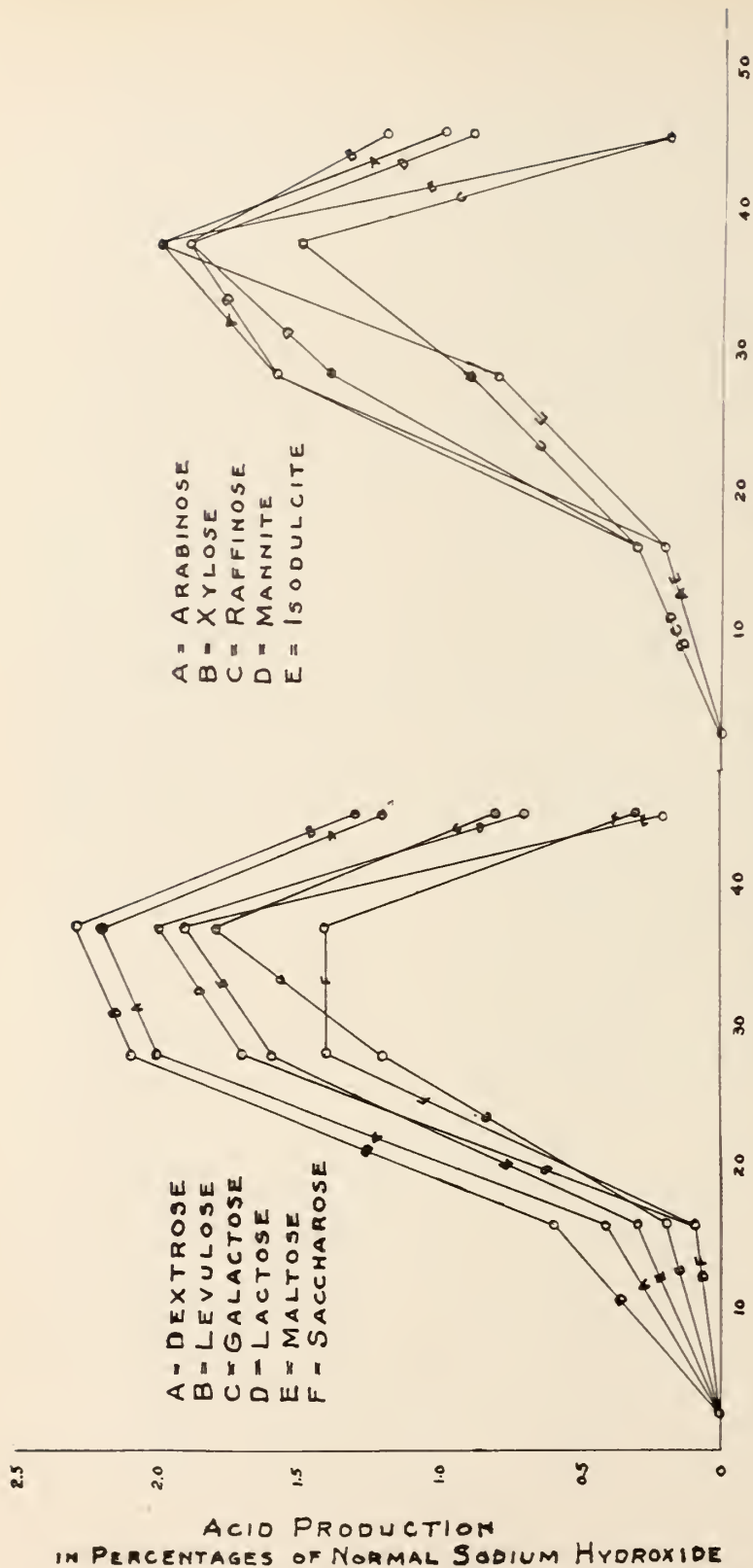


Chart 2.—The relation of temperature to the amount of acid produced by the bacillus coli, isolated from oysters.

or above, the amount of acid increases until the maximum amount of acid is produced at 37 C. We should expect this, since 37 C. is the optimum temperature for the growth of the bacillus coli group. If the temperature is too low or too high, we get less growth, and since acid production is dependent on the growth of the organism, we must surely find the optimum temperature for the production of acid identical with the optimum temperature for growth. This was found to be true in all the carbohydrate solutions used.

THE RELATION OF TIME TO THE AMOUNT OF ACID PRODUCED IN VARIOUS CARBOHYDRATE MEDIA

The general method of these experiments consisted in the inoculation of a large number of tubes of the 1 percent carbohydrate media with various members of the coli group. At definite intervals, a certain number of the tubes were removed from the incubator and titrated with N/20 sodium hydroxid. These experiments include 10 carbohydrates using various members of the bacillus coli group. Fifteen tubes of the 10 carbohydrates noted below, each containing 25 c.c. of the medium, were inoculated with 0.5 c.c. of a twenty-four-hour peptone culture of the bacillus coli. The tubes were incubated at 37 C., and were removed from the incubator and titrated at intervals of three hours.

From the results obtained in these experiments it is seen that, by the end of the twenty-fourth hour, the members of the bacillus coli group produce their maximum amount of acid when grown at 37 C., after the inoculation of a sugar medium with 0.5 c.c. of a twenty-four-hour peptone culture. In the case of some carbohydrates, such as dextrose or lactose, the maximum production of acid occurs in the eighteenth hour. The organisms used in this experiment produced their maximum amount of acid either before or at the twenty-fourth hour. Cultures, which were grown for 48 and 72 hours at 37 C., showed no increase over the amount of acid produced at the end of the twenty-fourth hour. The maximum amount of acid was produced by the organisms by the end of the twenty-fourth hour.

THE RELATION OF THE AMOUNT OF ACID PRODUCED TO THE AMOUNT OF MEDIUM INOCULATED

Erlenmeyer flasks, containing 25, 50, 100, 200, 300, 400, and 500 c.c. of dextrose and lactose broths, were inoculated with 0.5 c.c. of a twenty-four-hour culture of the coli bacillus. The flasks were incubated for 24 hours at 37 C., and then titrated with N/20 sodium hydroxid.

TABLE 3
ACID PRODUCTION, IN PERCENT NORMAL, AT INTERVALS OF THREE HOURS, BY THE *BACILLUS COLI*, ISOLATED FROM FECES

Hours	Percentage of Acid with Dextrose	Percentage of Acid with Lactose	Percentage of Acid with Saccharose	Percentage of Acid with Levulose	Percentage of Acid with Galactose	Percentage of Acid with Maltose	Percentage of Acid with Raffinose	Percentage of Acid with Arabinose	Percentage of Acid with Isodulcitate	Percentage of Acid with Mannite
3	1.0	0.6	0.35	0.95	0.35	0.7	0.35	0.5	0.35	0.35
6	1.9	1.4	0.3	1.8	0.7	0.8	0.7	1.7	1.1	1.1
9	2.2	1.9	0.4	1.9	0.85	1.05	1.15	1.9	1.6	1.3
12	2.35	1.9	1.1	2.1	1.3	1.7	1.3	2.0	1.9	1.3
15	2.4	1.9	1.85	2.2	1.9	1.8	1.35	2.1	1.9	2.0
18	2.4	1.9	2.0	2.3	1.9	2.0	1.7	2.2	2.2	2.0
21	2.4	1.9	2.0	2.3	2.0	2.0	2.0	2.2	2.2	2.0
24	2.4	1.9	2.0	2.2	2.1	2.0	2.1	2.2	2.1	2.0
42	2.4	2.0	2.1	2.3	2.1	2.0	1.9	2.2	2.1	2.0

All titrations are the average of titrations of two cultures.
The controls titrated at the end of 24 hours gave a neutral reaction.
The temperature was 37 C.

TABLE 4
ACID PRODUCTION, IN PERCENT NORMAL, AT INTERVALS OF THREE HOURS, BY THE *BACILLUS COLI*, ISOLATED FROM OYSTERS

Hours	Percentage of Acid with Dextrose	Percentage of Acid with Lactose	Percentage of Acid with Saccharose	Percentage of Acid with Levulose	Percentage of Acid with Galactose	Percentage of Acid with Maltose	Percentage of Acid with Raffinose	Percentage of Acid with Arabinose	Percentage of Acid with Isodulcitate	Percentage of Acid with Mannite
3	0.45	0.35	0.15	0.4	0.35	0.4	0.35	0.35	0.3	0.2
6	1.5	0.8	0.9	1.4	1.0	1.0	0.4	1.5	1.0	1.0
9	1.6	0.8	1.7	1.8	1.35	1.05	0.4	1.4	1.5	1.1
12	1.9	0.8	1.7	1.9	1.7	1.3	0.4	1.4	1.8	1.2
15	1.9	1.45	1.8	1.9	1.7	1.3	0.9	1.4	2.0	1.3
18	1.9	1.5	1.6	2.1	1.8	1.3	1.4	1.5	2.2	1.3
21	1.9	1.5	1.6	2.1	2.1	1.4	1.4	1.6	2.2	1.3
24	2.0	1.9	1.6	2.0	2.1	1.4	1.5	1.6	2.1	1.3
42	2.0	1.9	1.6	2.0	2.1	1.4	1.6	1.7	2.1	1.3

All titrations are the average of the titrations of two cultures.
The controls were titrated at the end of 24 hours and the reaction was neutral.
The temperature was 37 C.

The results obtained show that no matter how much carbohydrate media is inoculated, up to 500 c.c., the same percentage of acidity is produced by the bacillus coli when grown at 37 C. for 24 hours after inoculation with 0.5 c.c. of a twenty-four-hour peptone culture.

THE RELATION OF THE CONCENTRATION OF THE CARBOHYDRATE MEDIUM
TO THE AMOUNT OF ACID PRODUCED

Tubes of neutral nutrient broth, to which were added various percentages of dextrose and lactose, were inoculated with 0.5 c.c. of a twenty-four-hour peptone culture of the bacillus coli. The tubes were incubated for 24 hours at 37 C., and at the twenty-fourth hour the tubes were titrated with N/20 sodium hydroxid.

TABLE 5

AMOUNT OF ACID, IN PERCENT NORMAL, PRODUCED BY THE BACILLUS COLI ISOLATED FROM
FECES, IN NEUTRAL BROTH CONTAINING VARYING PERCENTAGES OF DEXTROSE AND LACTOSE

Percentage of Dextrose	Amount of Acid Produced Percent	Percentage of Lactose	Amount of Acid Produced Percent
0.125	0.4	0.125	0.35
0.25	0.5	0.25	0.5
0.5	2.1	0.5	1.7
0.1	2.2	1	1.8
1.5	2.2	1.5	1.8
2	2.3	2	1.8
2.5	2.2	2.5	1.75
3	2.4	3	1.8
3.5	2.3	3.5	1.8
4	2.2	4	1.8
4.5	2.3	4.5	1.8
5	2.4	5	1.8
7.5	2.35	7.5	1.7
10	2.3	10	1.8
15	2.3		
20	2.3		
25	2.3		
30	1.8		
35	0.9		
40	0.8		
45	0.6		
50	0.3		

The results are the average of the titrations of two cultures.
Temperature at 37 C.

From these experiments it appears that the concentration of the carbohydrates (dextrose and lactose) has little effect on the amount of acid produced, within certain limits, by the bacillus coli. Between 1 percent and 25 percent concentration, the amount of acid produced is nearly constant. Below 1 percent and above 25 percent there is less acid produced. A 1 percent solution gives the maximum amount of acid from the least amount of the carbohydrate.

THE RELATION OF THE INITIAL REACTION OF THE CARBOHYDRATE
MEDIUM TO THE AMOUNT OF ACID PRODUCED

The purpose of these experiments was to determine whether or not the initial reaction of the carbohydrate medium had any influence upon the production of acid by the organisms.

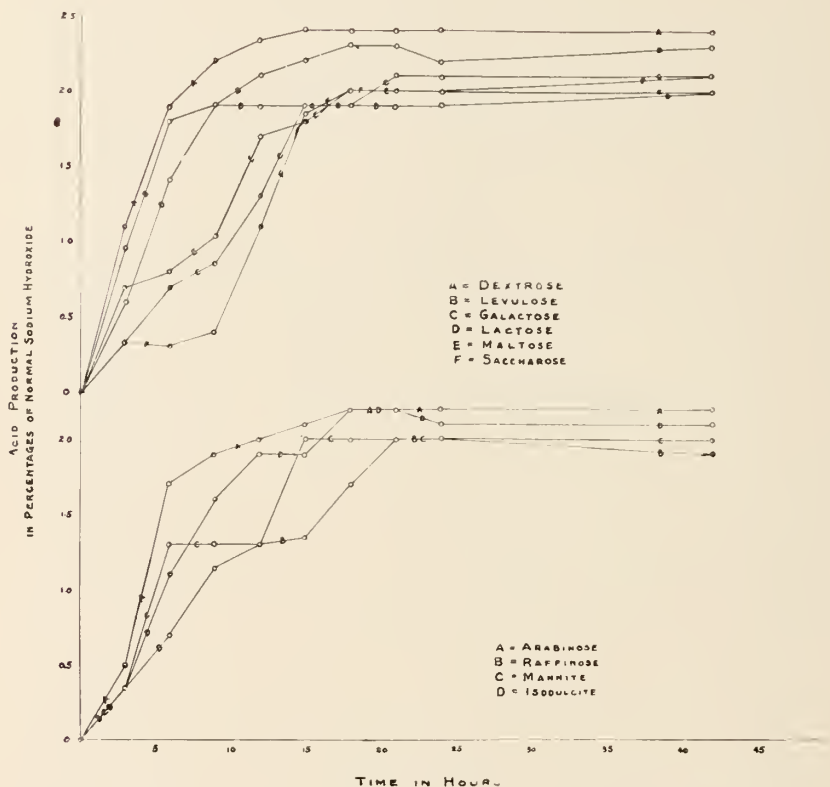


Chart 3.—The relation of time to the amount of acid produced by the bacillus coli, isolated from feces.

Tubes of dextrose broth, to which varying amounts of sterile acid and alkali were added after sterilization, were inoculated with 0.5 c.c. of a twenty-four-hour peptone culture of the bacillus coli. These tubes were incubated for 24 hours at 37 C. At the end of 24 hours the cultures were titrated with N/20 sodium hydroxid.

The amount of acid produced by the bacillus coli group, in various carbohydrate media, depends, in great part, upon the initial reaction of the medium. The maximum acid production of an organism is the

TABLE 6
 AMOUNT OF ACID, IN PERCENT NORMAL, PRODUCED IN DEXTROSE BROTH, WITH VARYING INITIAL REACTIONS, BY THE *BACILLUS COLI*,
 ISOLATED FROM FECES AND FROM OYSTERS

Initial Reaction of Dextrose Broth	Amount of Acid Produced by Colon Bacilli from Feces					Amount of Acid Produced by Colon Bacilli from Oysters				
	Culture				Control	Culture				Control
	1	2	3	Average		1	2	3	Average	
+4.4	4.4	4.5	4.4	4.42	4.3	4.3	4.3	4.3	4.3	+4.4
+2.65	2.6	2.7	2.7	2.66	2.65	2.5	2.5	2.5	2.5	+2.6
+2.1	2.3	2.4	2.3	2.33	2.0	2.3	2.3	2.3	2.3	+2.1
+1.0	2.3	2.3	2.3	2.3	1.0	2.5	2.5	2.5	2.5	+1.0
+0.15	2.2	2.3	2.3	2.26	0.1	2.3	2.3	2.3	2.3	+0.15
-0.5	2.3	2.3	2.4	2.33	0.5	2.4	2.4	2.4	2.4	-0.5
-0.6	2.3	2.3	2.3	2.3	0.6	2.4	2.4	2.4	2.3	-0.6

Temperature was 37 C.

amount of acid necessary to prevent further growth. The farther away the initial reaction is from the maximum acid production, the more acid an organism can produce until it reaches the maximum. The limiting acidity of the bacillus coli group is about 2.4 percent normal sodium hydroxid. A medium with a slightly acid reaction offers a shorter path to travel to the maximum acidity than a medium

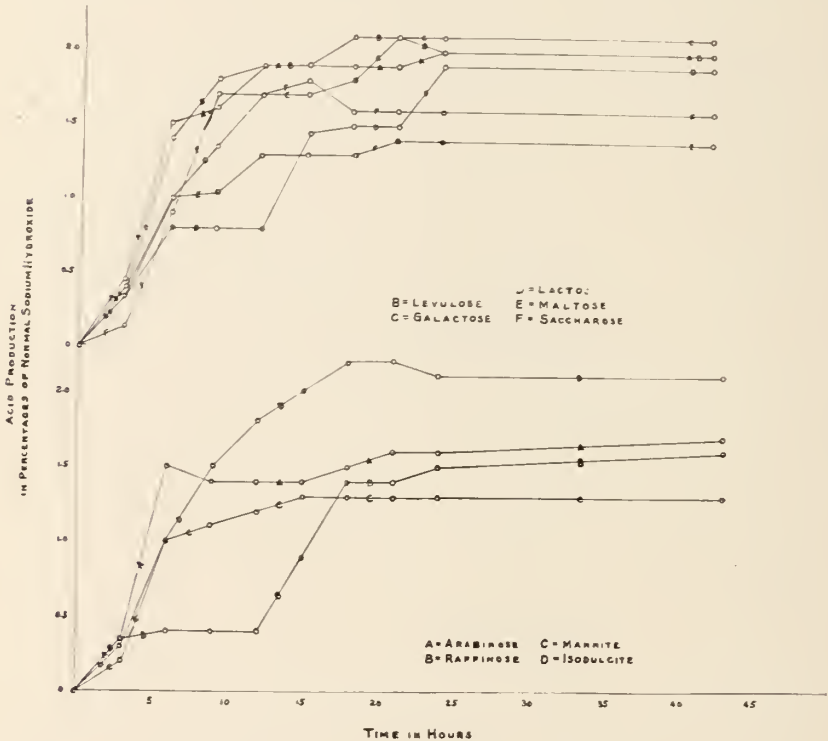


Chart 4.—The relation of time to the amount of acid produced by the bacillus coli, isolated from oysters.

with a neutral or slightly alkaline reaction. Hence, in a medium which is alkaline, the bacillus coli group will produce more acid than in a medium which is slightly acid, but in the end they will both reach the same goal—the maximum acidity of the organism. No acid is produced in a medium with over 2.4 percent acidity. The limit of alkalinity from which the organisms will produce acidity, was not determined.

TABLE 7

TOTAL AMOUNT ACID, IN PERCENT NORMAL, PRODUCED IN VARIOUS CARBOHYDRATES BY THE *BACILLUS COLI*, ISOLATED FROM FECES

Days	Percentage of Acid with Dextrose	Percentage of Acid with Levulose	Percentage of Acid with Galactose	Percentage of Acid with Lactose	Percentage of Acid with Maltose	Percentage of Acid with Saccharose	Percentage of Acid with Xylose	Percentage of Acid with Arabinose	Percentage of Acid with Raffinose	Percentage of Acid with Mannite	Percentage of Acid with Isodulcite
1	3.0	3.2	2.7	2.3	2.7	1.7	2.0	2.7	2.7	2.6	2.9
2	2.9	2.9	2.1	2.5	2.3	2.7	2.4	2.8	1.4	2.7	2.6
3	3.0	3.7	2.5	2.7	2.3	2.2	2.4	2.5	2.7	1.3	2.9
4	2.9	3.1	2.2	2.8	2.7	2.5	2.8	2.8	2.2	2.5	1.4
5	3.2	1.1	2.3	3.9	1.3	2.3	4.0	1.0	2.0	1.6	1.1
6	2.6	2.1	2.5	2.5	0.6	3.7	2.8	2.0	2.0	1.0	1.2
7	0.3	0.3	0.3	0.4	0	1.6	0	0.25	1.0	0	0
8	1.3	0.6	1.1	2.1	0.4	0.4	0	1.6	1.8	0	0.8
9	0.3	0.3	0.2	0.3	0	1.5	0	0.2	0.8	0	0
10	0.3	0.3	0.5	0.8	0.5	0.5	0	0.4	0.5	0.2	0.6
11	0.2	0.2	0.3	0.3	0	1.35	0	0	0.4	0.4	0
12	0.6	0.3	0.3	0.4	0.2	0.8	0	0	0.4	0.2	0.8
13	0.2	0	0.3	0.2	0	0	0	0	0.3	0	0
14	0.4	0.2	0.3	0.4	0.7	0.5	0	0	0.3	0.2	0.8
15	0	0	0	0.4	0	0.5	0	0	0.2	0	0
16	0	0	0.6	0	0	0	0	0	0	0	0.4
17	0	0	0	0	0	0.5	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0.1
19	0	0	0	0	0	0.2	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0
Total Average	10.6	9.15	8.95	11.0	6.85	11.7	8.2	7.62	9.45	6.35	7.3

TABLE 8

COMPARISON BETWEEN TOTAL ACID PRODUCTION AND THE MAXIMUM ACID PRODUCTION OF 24 HOURS INCUBATION

	Dextrose	Levulose	Galactose	Arabinose	Xylose	Mannite	Isodulcite	Lactose	Maltose	Saccharose	Raffinose
Total acid production	10.6	9.15	8.95	7.6	8.2	6.35	7.3	11.0	6.85	11.27	9.45
Maximum acid production of 24 hours	2.35	2.25	1.97	1.87	1.90	2.00	1.90	1.85	2.05	1.35	1.47

ACID PRODUCTION IN A MEDIUM PERIODICALLY NEUTRALIZED TO REMOVE THE ACIDS FORMED

Erlenmeyer flasks containing 100 c.c. of the various carbohydrates (1 percent concentration) were inoculated with 0.5 c.c. of a twenty-four-hour peptone culture of the different members of the bacillus coli group. Before inoculations were made, 0.5 c.c. of phenolphthalein (5 gm. of the commercial salt to one liter of 5 percent alcohol) was added as an indicator. The flasks were incubated at 37 C. and at intervals of 24 hours, the flasks were removed and sterile normal sodium hydroxid was added from a burette, until a faint pink color was obtained. The cultures were replaced in the incubator and the process was repeated at the end of every 24 hours, until no more acid was produced by the organisms.

Table 8 shows that the maximum acid production of 24 hours, which is, as previously proved, the greatest amount which the organism can produce at any one time, is limited by the excess of acid formed, and can be greatly increased by periodical neutralization.

The maximum amount of acid, which the bacillus coli group is able to produce in 1 percent carbohydrate solutions in 24 hours can be greatly increased if the excess of acid formed is neutralized. In all carbohydrates of 1 percent concentration, the total acid production is from 3 to 6 times as large as the maximum twenty-four-hour production which, as previously proved, is the same for 48 and 72 hours under ordinary conditions. From these experiments it may be assumed that acid production goes on until a maximum is reached (24 hours) and then ceases until some of the acid present is neutralized, when acid is again produced until the maximum is reached. This same thing occurs until all the carbohydrates are used. None of the cultures on the twelfth day gave a positive reaction for sugar, showing that the carbohydrates had been used entirely by the organisms in the production of acid. The total amount of acid an organism is able to produce, if the excess acid is neutralized, depends, in a large part, on how much fermentable carbohydrate there is present in the medium. The maximum twenty-four-hour production on the other hand, will use only that amount which is necessary to produce the maximum amount of acid which can be tolerated.

THE AMOUNT OF ACID PRODUCED IN VARIOUS CARBOHYDRATES

An attempt has been made to draw a comparison between the various carbohydrates by the amount of acid produced from them by different members of the bacillus coli group.

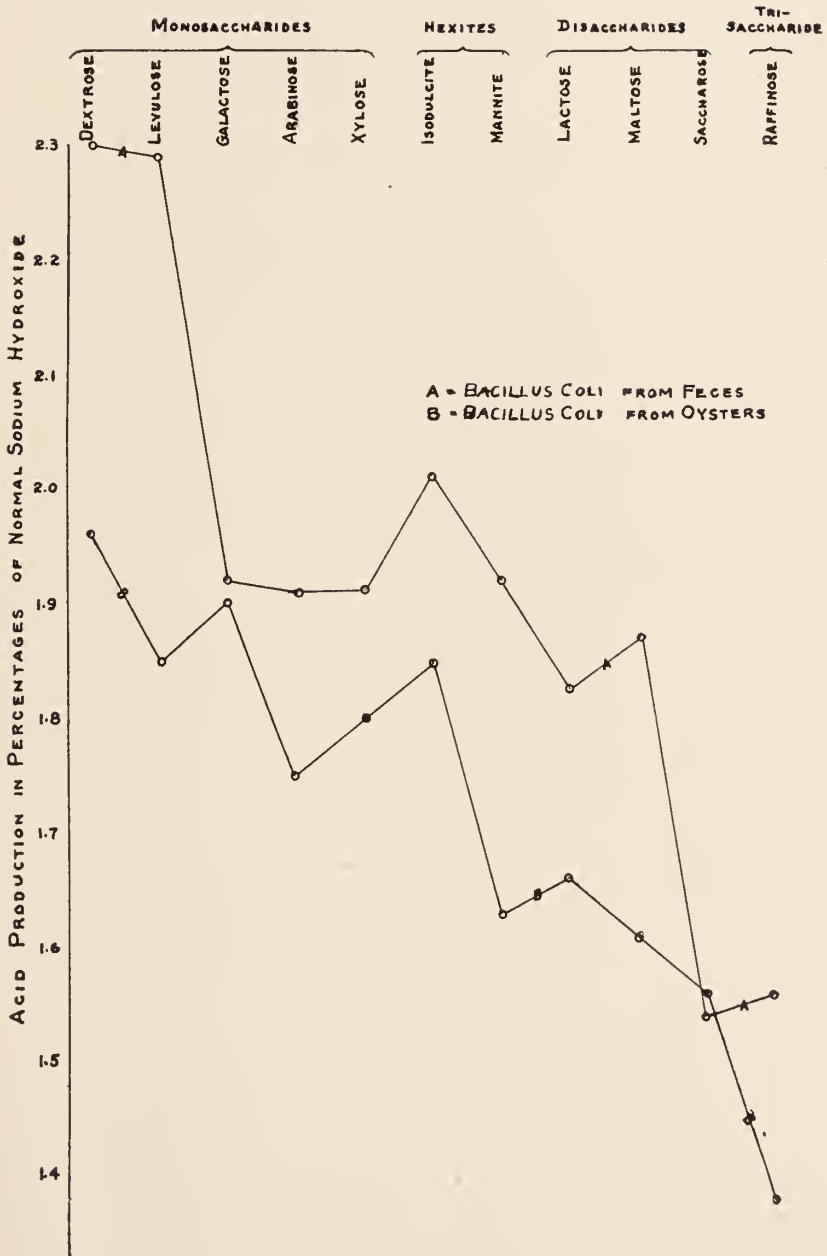


Chart 5.—The amount of acid produced in various carbohydrates by the *Bacillus coli*, isolated from feces and from oysters.

Tubes containing 25 c.c., of the various carbohydrates, were inoculated with 0.5 c.c. of a twenty-four-hour culture of the different members of the bacillus coli group. After 24 hours incubation at 37 C., the cultures were titrated with N/20 sodium hydroxid. In each case strains were selected which could ferment the carbohydrate in question.

Tables 9 and 10 show that the colon bacillus, whether isolated from feces or oysters, produces the maximum amount of acid in the monosaccharids, less in the disaccharids, and the least in the trisaccharid.

The results obtained in this series of experiments show that the various members of the bacillus coli group are able to produce more acid in solutions containing carbohydrates of simple chemical structure than in solutions containing carbohydrates of complex chemical structure. For instance, more acid is produced in solutions containing the monosaccharids and hexites, such as dextrose, levulose, galactose, xylose, arabinose, mannite, and isodulcite than in solutions containing the disaccharids, lactose, maltose, and saccharose. The disaccharids are, in turn, more easily fermented by the bacillus coli group than the trisaccharid raffinose.

The polysaccharid starch is not fermented by any of the members of the coli group. The trisaccharid raffinose, while not so complex a molecule as starch, appears to offer chemically greater difficulties to the organisms in the carrying out of their oxidative processes in the formation of the various acids from the sugars than the organisms would encounter when fermenting, for instance, a disaccharid. In fact, there are members of this group which are unable to ferment raffinose at all.

The arrangement of the carbohydrates, according to the amounts of acid which the members of the bacillus coli group are able to produce from them, agrees somewhat with an arrangement made according to their chemical complexity of structure, as may be seen by the following:

Monosaccharids, 2.03, 1.82.

Disaccharids, 1.74, 1.61.

Trisaccharid, 1.56, 1.38.

Polysaccharid (Starch), 0, 0.

Among the monosaccharids, the hexoses, dextrose, and levulose, seem to be most easily oxidized by the organism with the formation of acid. No distinct difference could be noted in the amounts of acid produced in the levulose and dextrose by the methods used in this

investigation. The amount of acid produced for these sugars also was very constant. Galactose, while not forming so much acid as dextrose and levulose, showed a greater variance in the amount of acid produced. All organisms investigated produced acid in the monosaccharids mentioned.

TABLE 10
ACID, IN PERCENT NORMAL, PRODUCED IN VARIOUS CARBOHYDRATES BY *BACILLUS COLI*, ISOLATED FROM OYSTERS

Carbohydrates	Cultures						Average
	1	2	3	4	5	6	
Dextrose	2.0	1.9	1.8	2.0	2.2	1.9	1.96
Levulose	1.9	1.6	1.7	1.6	2.2	2.1	1.85
Galactose	2.0	1.9	1.8	2.0	1.9	1.8	1.9
Arabinose	1.8	1.8	1.9	1.5	1.75
Xylose	1.8	...	1.8	1.8	...	1.8	1.8
Isodulcite	1.7	1.9	1.9	1.6	1.8	2.2	1.85
Mannite	2.0	1.5	1.6	1.6	1.8	1.3	1.63
<hr/>							
Average of all Monosaccharids and Hexites	1.82
<hr/>							
Lactose	1.9	1.6	1.6	1.8	1.8	1.3	1.66
Maltose	1.9	1.5	2.0	1.4	1.6	1.3	1.61
Saccharose	1.7	1.2	1.6	1.5	1.8	1.6	1.56
<hr/>							
Average of all Disaccharids	1.61
<hr/>							
Raffinose	1.2	1.7	1.4	0	1.2	1.4	1.38
<hr/>							
Average of Trisaccharid.	1.38

Tables 9 and 10 show that the colon bacillus, whether isolated from feces or oysters, produces the maximum amount of acid in the monosaccharids, less in the disaccharids, and least in the trisaccharid.

The pentoses, xylose and arabinose, seem to offer greater resistance to the bacterial oxidation processes, as we find less acid produced from the pentoses than from the hexoses. Throughout the work, these two sugars behaved exactly alike, and no differentiation was possible by the amount of acid produced from them by the group.

The hexites, mannite and isodulcite, showed less acid than the hexoses, and at times the amount of acid produced from them exceeded that produced from the pentoses. Many strains of the bacillus coli group were found, which were unable to ferment mannite and isodulcite.

Of the three disaccharids used, saccharose seems to offer greater resistance to the oxidative processes of the colon group than either lactose or maltose. In fact, many members of the group are unable to ferment it. This seems to be in accord with the chemical structure of the sugar, since we know that saccharose is the only one of the three mentioned disaccharids which cannot be oxidized. At times, the amount of acid produced in lactose and maltose equals or even exceeds the amount of acid produced in galactose, a monosaccharid.

The more complicated raffinose offers great difficulties to the fermentative processes of the bacillus coli group. Some strains failed to produce any acid at all from this sugar. As already mentioned, starch was not fermented by any strains used in this investigation.

It should not be understood, of course, that the size of the molecule is the only factor involved, for its configuration is also important. Winslow and Walker (1907) have shown that bacilli of the colon group, which ferment saccharose, usually ferment raffinose as well. The same thing appears in my results as to the amount of acid formed, saccharose results being lower than lactose results tho not quite so low as those obtained in raffinose.

There is an important distinction to be drawn between the power to ferment a given sugar and the amount of acid formed when it is fermented. In my work I have used only strains capable of attacking the sugar in question. When a low result is obtained it may be due to a slow action upon the sugar, or, in view of the evidence that it is the amount of end product formed which usually stops the reaction, it may be that the lower acidity produced in more complex sugars is the result of some other decomposition products, which accompany the acids, and, in connection with them, are able to inhibit growth.

ACOMPARISON BETWEEN THE AMOUNT OF ACID PRODUCED BY VARIOUS
MEMBERS OF THE BACILLUS COLI GROUP ISOLATED FROM
DIFFERENT SOURCES

In this series of experiments, a comparison has been made between the amounts of acid produced in various carbohydrates by the different members of the bacillus coli isolated from three distinct sources: (1) from stools of healthy individuals in and about the laboratory; (2) from stools of Italian immigrants quarantined on board the *S. S. Roma*; (3) from oysters taken from different locations in Narragansett Bay, representing areas of widely diverse characters.

The organisms were inoculated into tubes of peptone broth which were incubated at 37 C. for 24 hours. The various carbohydrates were inoculated by the addition of 0.5 c.c. of this twenty-four-hour culture. At the end of 24 hours' incubation at 37 C. the cultures were titrated with N/20 sodium hydroxid.

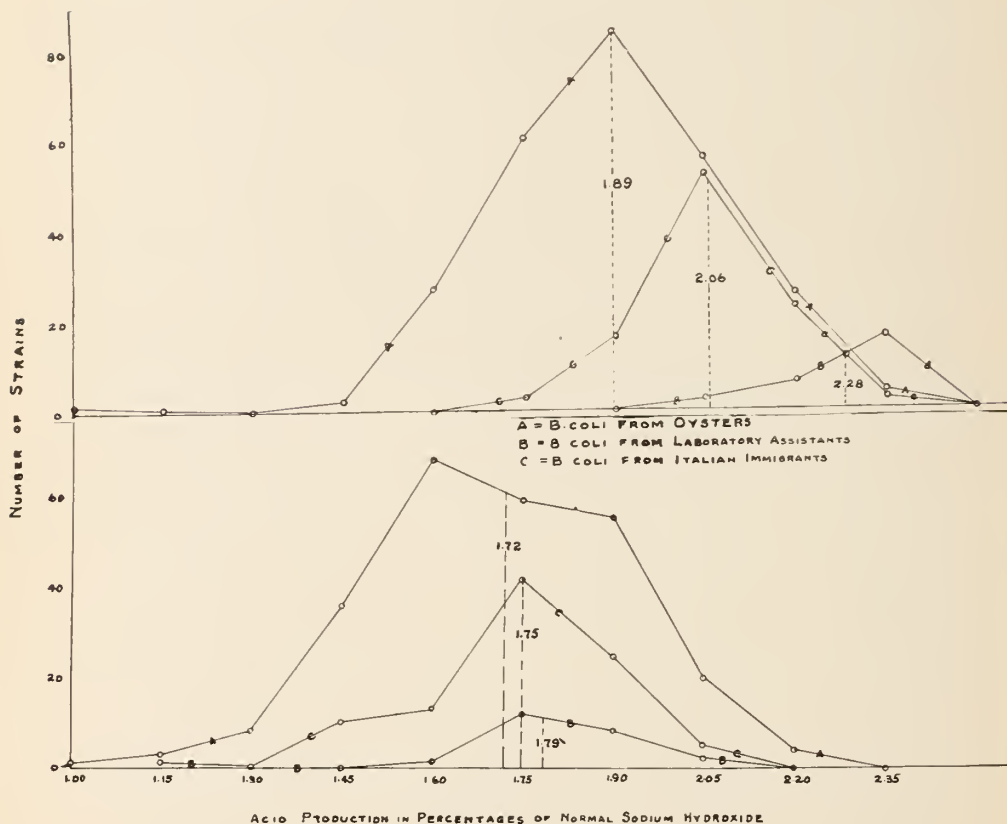


Chart 6.—The amount of acid produced by the bacillus coli in dextrose (upper curves) and lactose broth (lower curves).

The results obtained in this series of experiments seem to show that the source from which the members of the bacillus coli group were isolated has a direct effect upon the ability of the organisms to ferment carbohydrates with the production of acid.

A comparison between the amounts of acid produced in lactose and dextrose broths by members of the bacillus coli group gives the following results:

	From Laboratory Assistants	From Immigrants	From Oysters
Percentage in dextrose	2.28	2.06	1.89
Percentage in lactose	1.79	1.75	1.72

The bacillus coli isolated from feces, both from laboratory assistants and from the immigrants of the *S. S. Roma*, produced more acid in dextrose and lactose broth than the colon bacillus isolated from oysters. This seems to indicate that bacillus coli loses some of its ability to ferment carbohydrate with the production of acid during the journey from the intestinal tract to the oysters. Bacillus coli, isolated from the stools of laboratory assistants, produced more acid in dextrose and lactose broths than similar organisms isolated from the stools of Italian immigrants. The significance of this difference in the ability of the organisms, isolated from the above-named sources, to produce acid from carbohydrates means, is impossible to explain except that the general character of the diet may have had some effect on the ability of the organism to ferment carbohydrates with the production of acid.

EFFECT OF IMMERSION IN SEA WATER ON ABILITY TO PRODUCE ACID

The purpose of this experiment was to determine if the members of the bacillus coli group, after being kept for various periods in sea water, were affected in regard to their ability to produce acid in various carbohydrates.

In these experiments bottles of sea water, which gave two negative tests for the presence of the members of the bacillus coli group with lactose peptone bile, were inoculated with known cultures of the coli group and kept at the following temperatures: ice-box, 4 C.; room, 20 C.

At intervals of a week, portions of the sea water were inoculated into lactose peptone bile tubes, from which the members of the bacillus coli group were isolated and identified. From twenty-four-hour peptone cultures of these organisms, inoculations were made into the various carbohydrate media and incubated 24 hours at 37 C. At the end of that period titrations were made with N/20 sodium hydroxid.

TABLE 11
AMOUNT OF ACID, IN PERCENT NORMAL, PRODUCED IN DEXTROSE AND LACTOSE BROTH BY COLON BACILLI FROM
DIFFERENT SOURCES

Source and Number of Cultures	Dextrose			Lactose		
	Lowest	Highest	Average	Lowest	Highest	Average
Italian immigrants, 95	1.75	2.30	2.06	1.40	1.95	1.75
Laboratory assistants, 24	2.10	2.40	2.28	1.20	2.0	1.79
Oysters, 260	1.00	2.40	1.89	1.10	2.25	1.72

TABLE 12
AMOUNT OF ACID PRODUCED IN VARIOUS CARBOHYDRATE MEDIA BY THE BACILLUS COLI RETAINED IN SEA WATER AT 4 C. AND 20 C.

Weeks	Dextrose		Levulose		Galactose		Arabinose		Xylose		Mannite		Lactose		Maltose	
	4 C.	20 C.	4 C.	20 C.	4 C.	20 C.	4 C.	20 C.	4 C.	20 C.	4 C.	20 C.	4 C.	20 C.	4 C.	20 C.
1	2.3	2.3	2.3	2.3	1.2	1.2	1.8	1.8	1.9	1.8	2.0	2.0	1.7	1.8	1.6	1.5
2	2.3	2.2	2.25	1.9	1.4	1.2	1.8	1.7	1.7	1.8	2.1	2.0	1.7	1.6	1.5	0.4
3	2.3	2.2	2.3	2.3	1.1	1.2	1.8	1.7	1.7	1.8	2.0	2.0	1.7	1.6	1.5	1.9
4	4.25	2.2	2.3	2.2	1.6	1.6	1.8	1.9	1.95	1.9	2.0	2.1	1.85	1.7	1.7	1.7
5	2.0	2.1	2.3	2.1	1.6	1.4	1.7	1.3	1.9	1.9	1.9	1.9	1.6	1.8	0.6	0.8
6	2.2	2.2	2.3	2.0	1.5	1.7	1.6	2.0	1.9	1.8	2.1	2.0	1.7	1.8	1.8	1.9
7	2.3	1.4	2.3	1.7	1.9	1.6	1.6	2.0	1.9	1.8	1.8	1.7	1.7	1.7	1.8	1.9
8	2.2	...	2.3	...	1.2	...	1.6	...	1.9	...	2.0	...	1.5	...	1.7	...

All results are the averages of two titrations.

These experiments show that the various members of the bacillus coli group, after remaining for 8 weeks in bottles of sea water kept at 4 C. and 20 C., were able to produce the same amount of acid in the various carbohydrates as when first added.

SUMMARY

The optimum temperature for the maximum production of acid in 24 hours by the members of the bacillus coli group, in a medium containing a fermentable carbohydrate, is 37 C. Acid production in this time is almost nil at 3 C., rises rapidly to 37 C., and falls as rapidly above that point, ceasing between 50 C. and 60 C.

Twenty-four hours' incubation at 37 C. is a sufficient period for the maximum production of acid by members of the coli group in a medium containing a fermentable carbohydrate, when 0.5 c.c. of a twenty-four-hour peptone culture is used as an inoculum. Under these conditions no further increase in acidity occurs after 20 hours.

The amount of medium, up to 500 c.c., inoculated with the members of the coli group, has no effect upon the percentage of acidity produced by the organisms, when incubated at 37 C. for 24 hours after inoculation with 0.5 c.c. of a twenty-four-hour peptone culture.

A medium containing 1 percent of fermentable carbohydrate offers a suitable amount of carbohydrate for the maximum acid production by members of the coli group. A medium of much less than 1 percent concentration does not contain enough carbohydrate to bring about the maximum reaction, while a medium of more than 1 percent concentration serves no useful purpose. A high concentration of carbohydrates (over 25 percent) will not only hinder but will prevent the production of acid, but between 1 and 25 percent the twenty-four-hour acid production is the same.

The more distant the initial reaction of the medium is from the maximum acidity necessary to inhibit production of acid, the greater amount of acid the organism can produce before that maximum is reached. The extreme limit of alkalinity, in which the organism will produce acid, was not determined.

The maximum amount of acid, which bacilli of the colon group will produce in ordinary carbohydrate media, is fixed by the tolerance of the bacteria to the acid itself. If the acid formed is neutralized daily by the addition of free alkali, the acid production will go on until all the carbohydrate present has been consumed. With 1 percent carbo-

hydrate in the medium, there will be 4 or 5 times as much acid formed as ordinarily.

The members of the coli group produce the greatest amount of acid in media containing the monosaccharids and hexites, dextrose, levulose, galactose, arabinose, xylose, mannite, and isodulcite; less in the disaccharids, maltose, lactose, and saccharose; and least in the trisaccharid, raffinose.

Members of the bacillus coli group, isolated from feces, produce more acid in media containing fermentable carbohydrates than strains of the bacillus coli group, isolated from oysters taken from different portions of Narragansett Bay. Of the members of the bacillus coli group isolated from feces, strains which were obtained from the stools of laboratory assistants produced more acid in media containing fermentable carbohydrates than strains isolated from the stools of Italian immigrants quarantined aboard the *S. S. Roma*.

The amount of acid produced in various carbohydrate media by the bacilli of the colon group is unaffected by storage in unsterilized sea water for a period of 8 weeks at temperatures of 20 C. and 40 C.

GENERAL INDEX

SUBJECT INDEX

	PAGE
Acid-Fast Bacteria, Studies in, I-X - - - - -	417
Acid Fuchsin, Use of Decolorized, as an Acid Indicator in Carbohydrate Fermentation Tests with Some Remarks on Acid Production by Bacteria - - - - -	227
Acid Production by Bacteria, Some Remarks on - - - - -	227
Acid, Production of, by the Bacillus Coli Group - - - - -	580
Aciduric Bacteria, an Investigation into the Fermentative Activities of -	141
Allergic Reaction, Intracutaneous, and the Symptoms of Anaphylaxis, the Relation Between - - - - -	541
Anaerobic Cultures in Scarlet Fever - - - - -	85
Anaerobic Vibrio Isolated from a Case of Acute Bronchitis - - - - -	350
Anaphylaxis, The Relation Between the Allergic Intracutaneous Reaction and the Symptoms of - - - - -	541
Anilin Stains in Association with "Regressive Mordanting," Histologic Differentiation by Means of, with Special Reference to Elastic Tissue	561
Antibodies, The Formation of, in Rats Fed on Pure Vegetable Proteins (Osborne-Mendel Stunting Food) - - - - -	279
Antigenic Properties of Glycoproteins - - - - -	501
Anti Hog-Cholera Serum, Standardization of - - - - -	257
Antistreptococcus, Concentrated, Serum in Experimental Streptococcal Arthritis, Action of Vaccines and of - - - - -	215
Antitetanic Serum, The Treatment of Tetanus by - - - - -	367
Arthritis, Experimental, Streptococcal, Action of Vaccines and Concen- trated Antistreptococcus Serum in - - - - -	215
Arthritis, Infectious, of Colts, The Bacillus Abortus Equinus as an Etiological Factor in - - - - -	347
Arthritis, Pyemic, in Foals, The Etiology of - - - - -	409
Bacilli, A Simple Method of Cultivating, Preferring Conditions of Partial Anaerobiosis (B. Abortus, Bang; B. Bifidus, Tissier) - - - - -	22
Bacilli, Certain Acid-Fast, in Nutrient Broths, A Comparison of the Curves of Lipolytic Activity and Proteolysis of - - - - -	467
Bacilli, Certain Rapidly Growing Human Tubercle, in Broth Free from Lipoids and Fatty Substances, The Metabolism of - - - - -	423
Bacilli, Certain Rapidly Growing Human Tubercle, Metabolism of, in a Modified Uschinsky Medium - - - - -	428
Bacilli, Certain Rapidly Growing Tubercle, A Comparison of the Curves of Lipolytic Activity and Proteolysis of, in Media of Varied Compo- sition - - - - -	460
Bacilli, Certain Rapidly Growing Tubercle, Metabolism of, in Media with Inorganic Salts as Sources of Nitrogen - - - - -	433

	PAGE
Bacilli, Fat-Free, Tubercle and Granulation Tissue, An Experimental Study of the Influence of Iodin and Iodids on the Absorption of	487
Bacilli, Saphrophytic Human Tubercle, Metabolism of, in Plain, Dextrose, Mannite, and Glycerin Broths	417
Bacilli, Tubercle, and Other Acid-Fast Bacteria, Occurrence of a Soluble Lipase in Broth Cultures of	443
Bacilli, Tubercle, Certain Rapidly Growing Tubercle, The Relative Activity of Soluble Lipase and Lipase Liberated During Autolysis of	451
Bacillus Abortivus Equinus as an Etiological Factor in Infectious Arthritis of Colts	347
Bacillus Abortus, Bang; B. Bifidus, Tissier, A Simple Method of Cultivating Bacilli, Preferring Conditions of Partial Anaerobiosis	22
Bacillus Bifidus, Tissier; B. Abortus, Bang, A Simple Method of Cultivating Bacilli, Preferring Conditions of Partial Anaerobiosis	22
Bacillus Coli Group, The Production of Acid by	580
Bacillus Mucosus in the Blood, Purpura Associated with	151
Bacillus, Rapidly Growing Tubercle, Observations on the Specificity and Thermostability of the Lipase Developed During the Growth of, in Media of Varied Composition	455
Bacillus Tetani, Tetanus Toxin, and Certain Laboratory Animals, The Effect of Gentian Violet on	566
Bacillus, Typhoid, The Esterase Activity of Plain and Culture Broths of	354
Bacteria, Acid-Fast, and Tubercle Bacilli, Occurrence of a Soluble Lipase in Broth Cultures of	443
Bacteria, Acid-Fast, Studies in, I-X	417
Bacteria, Acid Production by, Some Remarks on	227
Bacteria, Aciduric, An Investigation into the Fermentative Activities of	141
Bacteria, Especially the Streptococci, Experiments on the Variability of the Fermentative Reaction of	234
Bacteria, of the Colon Type Found in Bovine Feces, Characteristics of	99
Bacterial Metabolism, Studies in, XXXIX. The Esterase Activity of Plain and Dextrose Broth Cultures of the Typhoid Bacillus	354
Bacteriology of Vaginitis	283
Bacterium Tularensis, Infection of Man with	331
Biochemistry and Chemotherapy of Tuberculosis, Studies on, IX. Tuberculocidal Action of Certain Chemical Disinfectants	245
Biochemistry and Chemotherapy of Tuberculosis, Studies on, X. An Experimental Study of the Influence of Iodin and Iodids on the Absorption of Granulation Tissue and Fat-Free Tubercle Tissue	487
Biochemistry and Chemotherapy of Tuberculosis, Studies on, XI. Therapeutic Value of Copper and Its Distribution in the Tuberculous Organism	518
Biometrical Study of Mucosus Capsulatus Group	268
Blood, Purpura Associated with Bacillus Mucosus in	151
Blood-Cells of Vertebrates, Some Structural Transformations of	319
Bovine Feces, Characteristics of Bacteria of the Colon Type Found in	99
Bovine Mastitis, Experimental, Produced with Hemolytic Streptococci of Human Origin	135
Bronchitis, Acute, An Anaerobic Vibrio Isolated from a Case of	350

Carbohydrate Fermentation Tests, Use of Decolorized Acid Fuchsin as an Acid Indicator in - - - - -	227
Carbohydrate Serum Broth of Constant Composition for Use in the Study of Streptococci, Method for Making - - - - -	209
Chemotherapy and Biochemistry of Tuberculosis, Studies on, IX. Tuberculocidal Action of Certain Chemical Disinfectants - - - - -	245
Chemotherapy and Biochemistry of Tuberculosis, Studies on, X. An Experimental Study of the Influence of Iodin and Iodids on the Absorption of Granulation Tissue and Fat-Free Tubercle Bacilli - - - - -	487
Chemotherapy and Biochemistry of Tuberculosis, Studies on, XI. The Therapeutic Value of Copper and Its Distribution in the Tuberculous Organism - - - - -	518
Chlamydo spores in <i>Sporothrix Schenckii</i> , The Formation of - - - - -	483
Cholesterol, Effect of, on Phagocytosis - - - - -	472
Classification of the Colon Group, Studies on - - - - -	187
Colon Bacillus Group, The Production of Acid by - - - - -	580
Colon Group, Studies on the Classification of - - - - -	187
Colon Type, Characteristics of Bacteria of, Found in Bovine Feces - - - - -	99
Colts, The <i>Bacillus Abortivus Equinus</i> as an Etiological Factor in the Infectious Arthritis of - - - - -	347
Complement-Fixation in <i>Gonococcus</i> Infections - - - - -	6
Copper, The Therapeutic Value of, and Its Distribution in the Tuberculous Organism - - - - -	518
Culture, An Attenuated, of <i>Trypanosoma Brucei</i> - - - - -	24
Cultures, Anaerobic, in Scarlet Fever - - - - -	85
Dengue, Etiology of. An Attempt to Produce the Disease in the Rhesus Monkey by the Inoculation of Defibrinated Blood - - - - -	341
Disinfectants, Certain Chemical, Tuberculocidal Action of - - - - -	245
Esterase Activity of Plain and Broth Cultures of the Typhoid Bacillus - - - - -	354
Etiology of Dengue. An Attempt to Produce the Disease in the Rhesus Monkey by the Inoculation of Defibrinated Blood - - - - -	341
Feces, Bovine, Characteristics of the Bacteria of the Colon Type Found in - - - - -	99
Fermentation Reactions, Classification of Pathogenic Streptococci by - - - - -	63
Fermentative Activities of the Aciduric Bacteria, An Investigation into - - - - -	141
Foals, The Etiology of Pyemic Arthritis in - - - - -	409
Fuchsin Acid, Decolorized, Use of as an Acid Indicator in Carbohydrate Fermentation Tests with Some Remarks on Acid Production by Bacteria - - - - -	227
Gentian Violet, The Effect of, on the <i>Bacillus Tetani</i> , Tetanus Toxin, and Certain Laboratory Animals - - - - -	566
Glycoproteins, The Antigenic Properties of - - - - -	501
<i>Gonococcus</i> and <i>Staphylococcus Albus</i> from the Urethra in Plate Culture, Observations on the Growth of (A Criticism of Warden's Work) - - - - -	309
<i>Gonococcus</i> Infections, Complement Fixation in - - - - -	6
Granulation Tissue and Fat-Free Tubercle Bacilli, An Experimental Study of the Influence of Iodin and Iodids on the Absorption of - - - - -	487
Hexose Sugar in Normal Milk, The Bacteriological and Chemical Evidence of the Occurrence of - - - - -	357

	PAGE
Indicator, Acid, in Carbohydrate Fermentation Tests, Use of Decolorized Acid Fuchsin as an - - - - -	227
Infections of Man with Bacterium Tularensis - - - - -	331
Intestinal Obstruction, Toxins of - - - - -	124
Investigation into the Fermentative Activities of the Aciduric Bacteria -	141
Iodids and Iodin, An Experimental Study of the Influence of, on the Absorption of Granulation Tissue and Fat-Free Tubercle Bacilli -	487
Lepra Bacillus, Grass Bacillus, and Smegma Bacillus, The Metabolism of, in Plain, Dextrose, Mannite, and Glycerin Broths - - - - -	439
Lipase Developed During the Growth of a Rapidly Growing Tubercle Bacillus in Media of Varied Composition, Observations on the Specificity and Thermostability of - - - - -	455
Lipase Liberated During Autolysis of Certain Rapidly Growing Tubercle Bacilli and Soluble Lipase, The Relative Activity of - - - - -	451
Lipase, Soluble, Occurrence of, in Broth Cultures of Tubercle Bacilli and Other Acid-Fast Bacteria - - - - -	443
Longevity, The Relative, of Different Streptococci and Possible Errors in the Isolation and Differentiation of Streptococci - - - - -	293
Lyperosia Irritans, An Attempt to Transmit Poliomyelitis by the Bite of	1
Mastitis, Experimental Bovine, Produced with Hemolytic Streptococci of Human Origin - - - - -	135
Metabolism, Bacterial, Studies in, XXXIX. The Esterase Activity of Plain and Dextrose Broth Cultures of the Typhoid Bacillus - -	354
Metabolism of Certain Rapidly Growing Human Tubercle Bacilli in a Modified Uschinsky Medium - - - - -	428
Metabolism of Certain Rapidly Growing Human Tubercle Bacilli in Broth Free from Lipoids and Fatty Substances - - - - -	423
Metabolism of Certain Rapidly Growing Tubercle Bacilli in Media with Inorganic Salts as Sources of Nitrogen - - - - -	433
Metabolism of "Lepra Bacillus," Grass Bacillus, and Smegma Bacillus in Plain, Dextrose, Mannite, and Glycerin Broths - - - - -	439
Metabolism of Saphrophytic Human Tubercle Bacilli in Plain, Dextrose, Mannite, and Glycerin Broths - - - - -	417
Milk and Milk Products, The Growth and Viability of Streptococci of Bovine and Human Origin in - - - - -	378
Milk, Infected, The Relation of Septic Sore Throat to - - - - -	130
Milk, Normal, The Bacteriological and Chemical Evidence of the Occurrence of a Hexose Sugar in - - - - -	357
Mucosus Capsulatus Group, A Biometrical Study of - - - - -	268
Nitrogen, The Metabolism of Certain Rapidly Growing Tubercle Bacilli in Media with Inorganic Salts as Sources of - - - - -	433
Organism, Pleomorphic Branching, Isolated from a Case of Chronic Rhinitis - - - - -	352
(Osborne-Mendel Stunting Food), The Formation of Antibodies in Rats Fed on Pure Vegetable Proteins - - - - -	279
Pathogenic Streptococci, Classification of, by Fermentation Reactions -	63
Phagocytosis, The Effect of Cholesterol on - - - - -	472
Pleomorphic Branching Organism Isolated from a Case of Chronic Rhinitis	352

Poison, Protein, of the Tonsil - - - - -	549
Poliomyelitis, An Attempt to Transmit, by the Bite of <i>Lyperosia Irritans</i> - - - - -	1
Pools, Swimming, Studies on the Sanitation of - - - - -	159
Protein Poison of the Tonsil - - - - -	549
Protein, Pure Vegetable (Osborne-Mendel Stunting Food), The Formation of Antibodies in Rats Fed on - - - - -	279
Purpura Associated with <i>Bacillus Mucosus</i> in the Blood - - - - -	151
Quinin Treatment of Rabies - - - - -	205
Rabbits Inoculated with Streptococci, The Renal Changes in - - - - -	389
Rabies, Quinin Treatment of - - - - -	205
Rats Fed on Pure Vegetable Proteins (Osborne-Mendel Stunting Food), The Formation of Antibodies in - - - - -	279
Reaction, Fermentative, of Bacteria, Especially the Streptococci Experiments on the Variability of - - - - -	234
Reactions, Fermentation, Classification of Pathogenic Streptococci by - - - - -	63
"Regressive Mordanting," in Association with Anilin Stains, Histologic Differentiation by Means of, with Special Reference to Elastic Tissue - - - - -	561
Rhinitis, Chronic, A Pleomorphic Branching Organism Isolated from a Case of - - - - -	352
Salts, Inorganic, as Sources of Nitrogen, The Metabolism of Certain Rapidly Growing Tubercle Bacilli in Media with - - - - -	433
Sanitation of Swimming Pools, Studies on - - - - -	159
Scarlet Fever, Anaerobic Cultures in - - - - -	85
Septic Sore Throat, The Relation of, to Infected Milk - - - - -	130
Serum, Anti Hog-Cholera, Standardization of - - - - -	257
Serum, Antitetanic, The Treatment of Tetanus by - - - - -	367
Sore Throat, Septic, The Relation of, to Infected Milk - - - - -	130
Sporothrix Schenckii, The Formation of Chlamydo spores in - - - - -	483
Standardization of Anti Hog-Cholera Serum - - - - -	257
Staphylococcus Albus and Gonococcus from the Urethra in Plate Culture, Observations on the Growth of - - - - -	309
Streptococci, Experiments on the Variability of the Fermentative Reaction of Bacteria, Especially - - - - -	234
Streptococci, Hemolytic, of Human Origin, Experimental Bovine Mastitis Produced with - - - - -	135
Streptococci, Method for Making Carbohydrate Serum Broth of Constant Composition for Use in the Study of - - - - -	209
Streptococci of Bovine and Human Origin in Milk and Milk Products, Growth and Viability of - - - - -	378
Streptococci, Pathogenic, Classification of, by Fermentation Reactions - - - - -	63
Streptococci, Possible Errors in the Differentiation and Isolation of - - - - -	293
Streptococci, The Relative Longevity of, and Possible Errors in the Isolation and Differentiation of - - - - -	293
Streptococci, The Renal Changes of Rabbits Inoculated with - - - - -	389
Swimming Pools, Studies on the Sanitation of - - - - -	159
Tests, Carbohydrate Fermentation, Use of Decolorized Acid Fuchsin as an Indicator in - - - - -	227
Tetanus, The Treatment of, by Antitetanic Serum - - - - -	367

Tetanus Toxin, <i>Bacillus Tetani</i> , and Certain Laboratory Animals, The	
Effect of Gentian Violet on - - - - -	566
Tonsil, The Protein Poison of - - - - -	549
Toxins of Intestinal Obstruction - - - - -	124
Treatment, Quinin, of Rabies - - - - -	205
<i>Trypanosoma Brucei</i> , An Attenuated Culture of - - - - -	24
Tubercle Bacilli and Other Acid-Fast Bacteria, Occurrence of a Soluble Lipase in Broth Cultures of - - - - -	443
Tubercle Bacilli, Certain Rapidly Growing Human, A Comparison of the Curves of Lipolytic Activity and Proteolysis of, in Media of Varied Composition - - - - -	460
Tubercle Bacilli, Certain Rapidly Growing Human, in a Modified Ushinsky Medium, Metabolism of - - - - -	428
Tubercle Bacilli, Certain Rapidly Growing Human, in Broth Free from Lipoids and Fatty Substances, Metabolism of - - - - -	423
Tubercle Bacilli, Certain Rapidly Growing Tubercle in Media with Inorganic Salts as Sources of Nitrogen - - - - -	433
Tubercle Bacilli, Certain Rapidly Growing, The Relative Activity of Soluble Lipase and Lipase Liberated During Autolysis of - - - - -	451
Tubercle Bacilli, Fat-Free, and Granulation Tissue, An Experimental Study of the Influence of Iodin and Iodids on the Absorption of - - - - -	487
Tubercle Bacilli, Saphrophytic Human, in Plain, Dextrose, Mannite, and Glycerin Broths, Metabolism of - - - - -	417
Tubercle Bacillus, a Rapidly Growing, Observations on the Specificity and Thermostability of the Lipase Developed During the Growth of, in Media of Varied Composition - - - - -	455
Tuberculocidal Action of Certain Chemical Disinfectants - - - - -	245
Tuberculous Organism, The Therapeutic Value of Copper and Its Distribution in the - - - - -	518
Typhoid Bacillus, The Esterase Activity of Plain and Dextrose Broth Culture of the - - - - -	354
Urethra, Observations on the Growth of the <i>Gonococcus</i> and the <i>Staphylococcus Albus</i> in Plate Cultures from the - - - - -	309
Ushinsky Medium, The Metabolism of Certain Rapidly Growing Human Tubercle Bacilli in a Modified - - - - -	428
Vaccine and Concentrated Antistreptococcus Serum, The Action of, in Experimental Streptococcal Arthritis - - - - -	215
Vaginitis, Bacteriology of - - - - -	283
Variability of the Fermentative Reaction of Bacteria, Especially the Streptococci, Experiments on - - - - -	234
Vertebrates, Some Structural Transformations of the Blood-Cells of - - - - -	319
<i>Vibrio</i> , Anaerobic, Isolated from a Case of Acute Bronchitis - - - - -	350
Warden's Work, A Criticism of - - - - -	309

INDEX OF AUTHORS

	PAGE
BEHRENS, CHARLES AUGUST - - - - -	24
BROWN, CLAUDE P. (KOLMER, JOHN A., and) - - - - -	6
BROWNE, WILLIAM W. - - - - -	580
BURMEISTER, W. H. - - - - -	549
CAPPS, JOSEPH A. (and DAVIS, DAVID J.) - - - - -	130
CAPPS, JOSEPH A. (DAVIS, DAVID J., and) - - - - -	135
CLARK, WILLIAM MANSFIELD (ROGERS, L. A., EVANS, ALICE C., and) -	99
CORPER, HARRY J. - - - - -	518
CRABTREE, E. G. - - - - -	309
CUMMINGS, JAMES GORDON - - - - -	205
DAVIS, DAVID JOHN - - - - -	378, 483
DAVIS, DAVID J. (and CAPPS, JOSEPH A.) - - - - -	135
DAVIS, DAVID J. (CAPPS, JOSEPH A., and) - - - - -	130
DAY, A. A. (KENDALL, A. I., WALKER, A. W., and) - - - - -	417
DEWEY, KÆTHE (and NUZUM, FRANK) - - - - -	472
DEWITT, LYDIA M. (and SHERMAN, HOPE) - - - - -	245
DICK, GEORGE F. (and HENRY, GLADYS R.) - - - - -	85
ELLIOTT, CHESTER H. - - - - -	501
EVANS, ALICE C. (ROGERS, L. A., CLARK, WILLIAM MANSFIELD, and) -	99
FITZGERALD, J. G. - - - - -	268
FRANCIS, EDWARD - - - - -	1
FRANCIS, EDWARD (LAVINDER, C. H., and) - - - - -	341
FRANKLIN, O. M. (HASLAM, THOMAS P., and) - - - - -	257
GOOD, EDWIN S. (and SMITH, WALLACE V.) - - - - -	347
GURD, FRASER B. - - - - -	124
HALL, IVAN C. (and TABER, LOREN B.) - - - - -	566
HARRIS, H. F. - - - - -	561
HASLAM, THOMAS P. (and FRANKLIN, O. M.) - - - - -	257
HEKTOEN, LUDVIG - - - - -	279
HENRY, GLADYS R. (DICK, GEORGE D., and) - - - - -	85
HOLMAN, W. L. - - - - -	209, 227, 293
HOPKINS, J. G. (and LANG, ARVILLA) - - - - -	63
HORTON, GEORGE D. - - - - -	22
HIRSCH, EDWIN FREDERICK - - - - -	487
IRONS, ERNEST E. - - - - -	367

	PAGE
JACKSON, LEILA (LeCOUNT, E. R., and)	389
JONES, HARRY M.	357
KENDALL, A. I. (and SIMONDS, J. P.)	354
KENDALL, A. I. (DAY, A. A., and WALKER, A. W.)	443
KENDALL, A. I. (WALKER, A. W., and DAY, A. A.)	417
KITE, G. L.	319
KLIGLER, I. J.	187
KOLMER, JOHN A. (and BROWN, CLAUDE P.)	6
LAMB, B. H. (WHERRY, WILLIAM B., and)	331
LANG, ARVILLA (HOPKINS, J. G., and)	63
LAVINDER, C. H. (and FRANCIS, EDWARD)	341
LeCOUNT, E. R. (and JACKSON, LEILA)	389
MANHEIMER, WALLACE A.	159
McMEANS, J. W. (WEIL, G. C., and)	151
MEIGS, GRACE L.	541
MOORE, JOSIAH J.	215
NUZUM, FRANK (DEWEY, KAETHE, and)	472
RAHE, ALFRED	141
ROGERS, L. A. (CLARK, WILLIAM MANSFIELD, and EVANS, ALICE C.)	99
SCHOFIELD, FRANK M.	409
SHARP, W. B.	283
SHERMAN, HOPE (DEWITT, LYDIA M., and)	245
SIMONDS, J. P. (KENDALL, A. I., and)	354
SMITH, WALLACE V. (GOOD, EDWIN S., and)	347
TABER, LOREN B. (HALL, IVAN C., and)	566
THRO, WILLIAM C.	234
TUNNICLIFF, RUTH	350, 352
WALKER, A. W. (KENDALL, A. I., DAY, A. A., and)	417
WEIL, G. C. (and McMEANS, J. W.)	151
WHERRY, WILLIAM B. (and LAMB, B. H.)	331

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